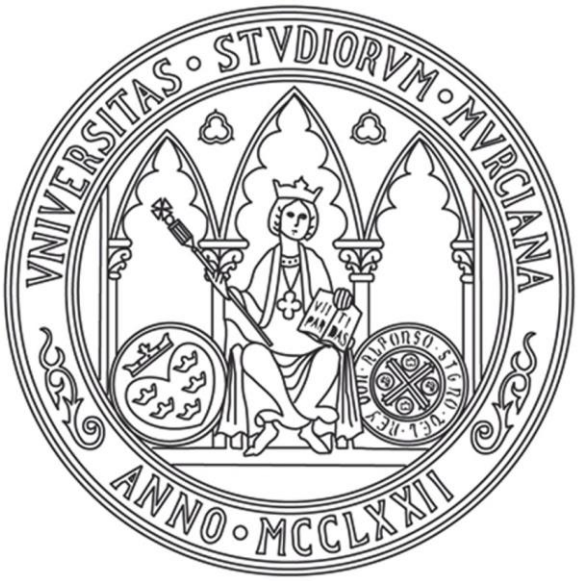

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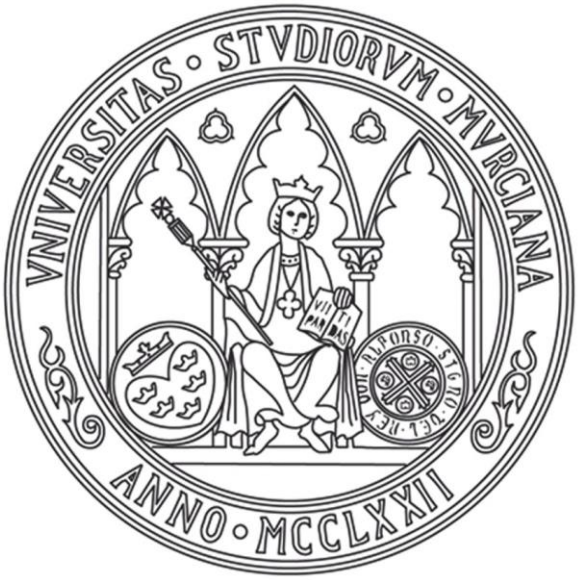
ESCUELA INTERNACIONAL DE DOCTORADO

Microbial biodiversity assessment of artificial microhabitats: examples from Egyptian archaeological sites

Evaluación de la biodiversidad microbiana de microhábitats artificiales: ejemplos de yacimientos arqueológicos egipcios

Dña. Samah Mohamed Rizk

2021



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Microbial biodiversity assessment of artificial microhabitats: examples from Egyptian archaeological sites

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Murcia, February 2021

Dedicated to my family,

Especially the two dearest persons

to my heart, who always filled

me unconditionally with love,

care, and support, my father 'God

bless his soul' and my husband.

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I counted all I remembered instantly, while others are in my heart forever even if I forgot to mention.

Thank you All…

Samah

TABLE OF CONTENTS

RESUMEN

Los yacimientos arqueológicos de piedra son una prueba inestimable de la presencia prehistórica del hombre en un lugar determinado de la tierra. Sin embargo, de manera inevitable están destinados a sufrir un lento proceso natural de transformación en arena y polvo que conduce a su pérdida, fenómeno denominado deterioro. Tales cambios son causados por factores físicos, químicos y biológicos. La degradación y deterioro de las construcciones de piedra crean un microhábitat heterogéneo caracterizado por la baja disponibilidad de agua y nutrientes. Este microbioma de piedras está compuesto por algas, hongos (incluidos líquenes) y bacterias de diversas afiliaciones filogenéticas, tales como Cyanobacteria, Actinobacteria, etc. La estrategia en la colonización de estos organismos varía desde la epilítica, la casmolítica y la endolítica, sin embargo, apenas sobreviven y rara vez se reproducen.

La presente tesis doctoral se centró en el estudio de las bacterias y hongos que habitan las piedras y rocas de yacimientos arqueológicos importantes, denominados por sus siglas en inglés, SIB (*stone-inhabiting bacteria*) y RIF (*rock-inhabiting fungi*). Dentro de los SIB, los fotótrofos facilitan el crecimiento de la microflora heterotrófica que posteriormente coloniza estos ambientes. Alternativamente, en las regiones áridas, los contaminantes orgánicos, el polvo y la suciedad favorecen el crecimiento directo de bacterias heterotróficas de naturaleza oligotrófica. Las bacterias filamentosas pueden causar daños estructurales en las piedras debido a que son capaces de penetrar en ellas, forman biopelículas y secretan enzimas y pigmentos oscuros solubles en agua. La flora fúngica de las rocas o RIF está compuesta por hongos meristemáticos microcoloniales (levaduras negras y hongos similares a las levaduras). Estos últimos generalmente producen células isodiamétricas hinchadas con paredes celulares gruesas que contienen melanina. Aunque los RIF suelen estar presentes en cantidades bajas, son altamente erosivos y pueden originar manchas y otras alteraciones estéticas significativas, grietas y fisuras en las rocas. Los RIF pueden permanecer metabólicamente activos incluso en condiciones de baja disponibilidad de nutrientes y tienen una alta resistencia a la desecación, la radiación UV y el estrés osmótico, incluso son capaces de sobrevivir en condiciones espaciales simuladas y reales sin

necesidad de luz. Por lo tanto, se adaptan bien al crecimiento en superficies rocosas y causan un grave problema al patrimonio cultural en todo el mundo.

La evaluación de los microorganismos asociados a los yacimientos del patrimonio cultural es particularmente difícil. El daño causado al extraer una muestra representativa debe sopesarse frente a la información que se va a obtener. Por tanto, es preferible usar métodos no destructivos y de micro muestreo. Estos microorganismos se han identificado tradicionalmente mediante técnicas de cultivo *in vitro*. La mayoría de las colecciones de cultivos se aislaron en medios ricos convencionales que no imitan el estado oligotrófico de los hábitats naturales. Sin embargo, con estas técnicas solo se detecta una pequeña fracción de la población total de una muestra ambiental, lo que lleva a una subestimación grave de la abundancia y diversidad microbiana. Una gran parte de los microorganismos presentes en los yacimientos de piedra al aire libre son oligo- o poiquilotróficos que crecen lentamente, pero son resistentes a las condiciones adversas, lo que les permite tolerar las duras condiciones en las superficies de la piedra. Por lo tanto, es importante elegir el medio de cultivo correcto para reducir el desarrollo de microbios de crecimiento rápido y prolongar el tiempo de incubación que permita el desarrollo de aquellos de crecimiento lento.

La aplicación de métodos moleculares en ecología permite realizar investigaciones independientes del cultivo de las comunidades microbianas. El ARN ribosómico (ARNr) está bien conservado desde el punto de vista evolutivo y contiene regiones hipervariables que permiten distinguir entre taxones estrechamente relacionados. El gen del ARNr 16S bacteriano y de arqueas, y las secuencias de ADNr (18S-5.8S-28S en conjunto con los espaciadores transcritos intergénicos (ITS) 1 y 2) en hongos son los genes usualmente elegidos para la identificación de estos organismos a nivel molecular. Las bases de datos de genes permiten identificar especies microbianas mediante enfoques metagenómicos. Los continuos avances en la metagenómica y la secuenciación de alto rendimiento han hecho que estos ensayos sean más aplicables a múltiples entornos, especialmente cuando se requiere revelar nuevos microbios independientes del cultivo. Asimismo, los avances en la tecnología de PCR permiten la cuantificación (qPCR) de la concentración de la

población microbiana utilizando el gen del ARNr 16S, y otros como el gen de la Beta-actina, el de la Celobiohidrolasa I (*cbh* I) y la región ITS. Todos ellos resultaron útiles en caracterizar la actividad y presencia de microorganismos en los yacimientos arqueológicos. Sin embargo, la exploración de más marcadores moleculares no es frecuente en el campo de la microbiología del patrimonio cultural y aún está en curso.

El objetivo general de esta tesis doctoral fue conocer la diversidad microbiana presente en microhábitats de yacimientos arqueológicos de elevado valor cultural, expuestos constantemente a condiciones ambientales duras y áridas. Se eligieron las pirámides de Djoser y Lahun, dos de las más antiguas y grandes de la necrópolis de Memphis del antiguo Egipto. Se prestó especial atención a los organismos extremófilos y extremotolerantes como SIB y RIF. Se realizaron estudios comparativos mediante análisis de metabarcoding basado en amplicones y métodos de aislamiento tradicionales de cultivo *in vitro*, así como técnicas de microscopía de epifluorescencia: Los objetivos específicos fueron: 1) conocer la flora microbiana que habita las pirámides de Djoser y Lahun, su riqueza específica, abundancia, filogenia y taxonomía; 2) estudiar si los microorganismos que habitan sobre sus superficies rocosas son potenciales agentes biodeteriorantes; 3) comparar la efectividad del análisis de metabarcoding y los métodos tradicionales de aislamiento para el conocimiento microbiano de las pirámides estudiadas; 4) caracterizar las cepas de especial interés halladas en cultivo mediante pruebas fisiológicas, químicas y de genotipificación de múltiples locus para definir sus límites de tolerancia a condiciones extremas y duras y sus relaciones filogenéticas; 5) establecer un protocolo rápido aplicable al campo de la microbiología del patrimonio cultural para la detección de SIB a menor costo basado en técnicas de “minería genómica” y qPCR; 6) explorar nuevos marcadores moleculares específicos de SIB.

El núcleo de la tesis doctoral consta de tres capítulos. Cada uno de ellos contiene un estudio sobre diferentes aspectos de la biodiversidad microbiana de las pirámides de Djoser y Lahun, especialmente los potenciales agentes causantes de biodeterioro que pueden habitar sobre ellas y degradarlas.

En el capítulo I se realizó el estudio microbiano comparativo de las pirámides de Djoser y Lahun mediante el uso combinado de métodos de metabarcoding, de microscopía de epifluorescencia y de cultivos *in vitro*. El análisis de metabarcoding identificó 644 especies de bacterias (452 géneros) utilizando el gen 16S del ADNr y 204 especies de hongos (146 géneros) utilizando la región ITS2. En los ensayos de aislamiento se identificaron 28 especies bacterianas (13 géneros) y 34 especies de hongos (21 géneros). Un total de 19 especies de bacterias y 16 de hongos dependían exclusivamente del cultivo, mientras que 92 especies de bacterias y 122 de hongos eran independientes del cultivo. Las SIB más abundantes fueron *Blastococcus aggregatus*, *B. saxobsidens* y *Blastococcus* sp. Otras bacterias frecuentes fueron *Bacillus alkalitelluris*, *B. persicus*, *Planococcus salinarum* y *Planococcus* sp. Los RIF más abundantes fueron *Knufia karalitana* y *Pseudotaeniolina globosa*, además de una especie desconocida de la familia Sporomiaceae. En ambas pirámides se detectaron microorganismos asociados con el biodeterioro. La diversidad microbiana fue mayor de lo esperado, considerando las duras condiciones de los sitios de muestreo. Se confirmó que el mejor enfoque metodológico para identificar y estudiar una comunidad microbiana compleja es a través de la combinación de microscopía e identificación molecular para microbios dependientes del cultivo y de métodos de metagenómica para aquellos independientes del cultivo. Este estudio es el primero que utiliza simultáneamente enfoques dependientes e independientes del cultivo para esas dos pirámides y pone de manifiesto la urgente necesidad de su conservación desde una perspectiva microbiológica.

En el capítulo II se llevó a cabo la caracterización morfológica, fisiológica y molecular de una cepa del hongo meristemático negro o RIF, *Pseudotaeniolina globosa* aislada en la pirámide de Djoser y se comparó con una cepa italiana obtenida en trabajos anteriores. Se realizaron experimentos para probar sus preferencias de temperatura, salinidad y pH, el nivel de tolerancia a la radiación UV y a alta temperatura, así como el genotipado multilocus usando cinco marcadores (ITS, nrSSU o 18S, nrLSU o 28S, BT2 y RPB2). Todos los datos confirmaron la coespecificidad de las dos cepas. Sin embargo, la egipcia fue capaz de crecer en un mayor rango de temperaturas

(4-37 °C) que la italiana (10-30 °C), a valores de pH más amplios (3,0-9,0 pH frente a 4.0-6,0 pH) y a niveles extremos de salinidad (5 M NaCl) frente a los más bajos de la italiana (0.20 M NaCl). Asimismo, la cepa egipcia mostró más tolerancia al calor ya que fue capaz de crecer tras la exposición hasta de 85 °C durante 5 min y la italiana solamente hasta 80 °C durante 5 min, así como a la radiación UV, ya que la tasa de crecimiento en la egipcia no se vio afectada hasta las 9 h de exposición, a diferencia de la italiana que sí le perjudicó. Sobre la base de los resultados filogenéticos, *P. globosa* se atribuyó al grupo de la familia Teratosphaeriaceae, orden Capnodiales. Nuestros resultados demostraron que la cepa egipcia se puede considerar una biovariante bien adaptada a entornos extremos y duros que actualmente coloniza la pirámide Djoser de Egipto. Su potencial efecto biodeteriorante sobre tan importante patrimonio cultural requiere atención para diseñar y buscar planes y soluciones de conservación para limitar su presencia y extensión en el área estudiada.

En el capítulo III se desarrollaron marcadores moleculares específicos para detectar de forma rápida y con bajo costo la presencia de Actinobacteria que viven sobre piedras (SIAb) en el patrimonio cultural antes de realizar análisis adicionales (p. ej., de metagenómica y metabarcoding). Los datos publicados de dos especies de SIB (*Blastococcus saxobsidens* y *Geodermatophilus obscurus*) se utilizaron para extraer sus genomas. La búsqueda se centró en la vía de la síntesis de aminoácidos similares a las micosporinas (MAAs). Estos aminoácidos son compuestos intracelulares biosintetizados por la vía del ácido siquímico que han sido relacionados con la resistencia abiótica en microorganismos. Mediante “minería genómica” se obtuvieron dos genes candidatos: un homólogo de un gen clave en los MAAs y las vías del siquimato conocido como DAHP II (*aro*F) y otro homólogo del gen Corismato mutasa (*cm*2). Ambos se encontraron principalmente en Actinobacteria. Después de la calibración con cinco especies de SIAb usando PCR convencional, los cebadores de nuevo diseño se aplicaron con éxito al ADN ambiental extraído de las pirámides de Djoser y Lahun utilizando qPCR. Hasta donde sabemos, este es el primer trabajo en el que se proponen *aro*F y *cm*2 como marcadores de qPCR para detectar SIAb. La elevada sensibilidad de la qPCR para detectar concentraciones muy bajas de ADN en muestras de

laboratorio y campo, la convierte en una herramienta muy útil para aislar especies de SIAb en yacimientos arqueológicos. El desarrollo de nuevos métodos de identificación permite un mayor conocimiento de la diversidad de organismos presentes en los monumentos al aire libre ya que puede llevar al descubrimiento de nuevas especies o biovariantes.

En el presente estudio, la combinación del enfoque de metabarcoding con el método de aislamiento tradicional ayudó a desarrollar de manera efectiva una estrategia en varios pasos para estudiar e identificar la diversidad de las comunidades microbianas presentes en las pirámides de Djoser y Lahun.

Futuros trabajos en esta línea de investigación deberían tener como objetivo aislar y caracterizar mediante metabarcoding especies con relaciones filogenéticas desconocidas. Asimismo, conocer las secuencias responsables de la extremotolerancia de la cepa egipcia de *P. globosa* sería de gran interés, para lo cual sería necesario realizar una comparación a nivel genómico, transcriptómico y metabólico utilizando enfoques basados en secuenciación de nueva generación. Finalmente, sería de gran interés la realización de más análisis bioinformáticos para desarrollar nuevos marcadores moleculares capaces de detectar aquellos grupos de microorganismos hallados en abundancia en las pirámides estudiadas y cuya identidad permanece desconocida.

ABSTRACT

Archaeological stone sites are invaluable proof of the prehistoric presence of man in a particular place on earth. However, they are inevitably destined to undergo a slow natural process of transformation into sand and dust that leads to their loss, a phenomenon called biodeterioration. Such changes are caused by physical, chemical, and biological factors. The degradation and deterioration of stone constructions create a heterogeneous microhabitat characterized by the low availability of water and nutrients. The stone microbiome is made up of algae, fungi (including lichens), and bacteria of various phylogenetic affiliations, such as Cyanobacteria, Actinobacteria, etc. The strategy in the colonization of these organisms varies from epilithic, chasmolithic and endolithic, however, they barely survive and rarely reproduce.

The current doctoral thesis focused on the study of bacteria and fungi that inhabit the stones and rocks of important archaeological sites, known by their acronyms in English, SIB (stone-inhabiting bacteria) and RIF (rock-inhabiting fungi). Within SIBs, phototrophs facilitate the growth of heterotrophic microflora that subsequently colonizes these environments. Alternatively, in arid regions, organic pollutants, dust and dirt favor the direct growth of heterotrophic bacteria of an oligotrophic nature. Filamentous bacteria can cause structural damage to stones because they are able to penetrate stones, form biofilms, and secrete enzymes and dark, water-soluble pigments. The rock fungal flora or RIF is composed of microcolonial meristematic fungi (black yeast and yeast-like fungi). The latter generally produce swollen isodiametric cells with thick cell walls that contain melanin. Although RIFs are usually present in low amounts, they are highly erosive and can cause staining and other significant aesthetic alterations, cracks and fissures in rocks. RIFs can remain metabolically active even in conditions of low-nutrient availability and have a high resistance to desiccation, UV radiation and osmotic stress, they are even able to survive in simulated and real space conditions without the need for light. Therefore, they adapt well to growth on rocky surfaces and cause a serious problem to cultural heritage throughout the world.

The assessment of microorganisms associated with cultural heritage sites is particularly difficult. The damage caused to collect a representative sample must be weighed against the information to be obtained. Therefore, it

is preferable to use non-destructive and micro-sampling methods. These microorganisms have traditionally been identified by *in vitro* culture techniques. Most of the culture collections were isolated on conventional rich media that do not mimic the oligotrophic state of natural habitats. However, these techniques only detect a small fraction of the total population of an environmental sample, leading to a severe underestimation of microbial abundance and diversity. A large part of the microorganisms present in outdoor stone deposits are oligo- or poikilotrophic, which grow slowly, but are resistant to adverse conditions, allowing them to tolerate harsh conditions on stone surfaces. Therefore, it is important to choose the correct culture medium to reduce the growth of fast-growing microbes and to prolong the incubation time to allow the development of slow-growing ones.

The application of molecular methods in ecology allows independent investigations of the culture of microbial communities. Ribosomal RNA (rRNA) is in general evolutionarily well conserved but also contains hypervariable regions that allow the distinction between closely related taxa. The bacterial and archaea 16S rRNA gene and rDNA sequences (18S-5.8S-28S in conjunction with intergenic transcript spacers (ITS) 1 and 2) in fungi are the genes usually chosen for the identification of these organisms at the molecular level. Gene databases allow the identification of microbial species using metagenomic approaches. Continued advances in metagenomics and high-throughput sequencing have made these assays more applicable to multiple settings, especially when new culture-independent microbes need to be revealed. Likewise, advances in PCR technology allow the quantification (qPCR) of the concentration of the microbial population using the 16S rRNA gene, and others such as the Beta-actin gene, that of Cellobiohydrolase I (cbh I) and the ITS region. All of them were useful in characterizing the activity and presence of microorganisms in archaeological sites. However, the exploration of more molecular markers is rare in the field of cultural heritage microbiology and is still ongoing.

The general objective of this doctoral thesis was to know the microbial diversity present in microhabitats of archaeological sites of high cultural value, constantly exposed to harsh and arid environmental conditions. The pyramids

of Djoser and Lahun were chosen, two of the oldest and largest in the Memphis necropolis of ancient Egypt. Particular attention was paid to the extremophilic and extremotolerant organisms such as SIB and RIF. Comparative studies were carried out using amplicon-based metabarcoding analysis and traditional *in vitro* culture isolation methods, as well as techniques of epifluorescence microscopy: The specific objectives were: 1) to know the microbial flora that inhabits the pyramids of Djoser and Lahun, its specific richness, abundance, phylogeny and taxonomy; 2) to study if the microorganisms that inhabit its rocky surfaces are potential biodeteriorating agents; 3) to compare the effectiveness of metabarcoding analysis and traditional isolation methods for microbial knowledge of the studied pyramids; 4) to characterize the strains of special interest found in culture by means of physiological, chemical and genotyping tests of multiple loci to define their tolerance limits to extreme and harsh conditions and their phylogenetic relationships; 5) to establish a rapid protocol applicable to the field of cultural heritage microbiology for the detection of SIB at a lower cost based on “genomic mining” techniques and qPCR; 6) to explore new molecular markers specific to SIB.

The core of the doctoral thesis consists of three chapters. Each of them contains a study on different aspects of the microbial biodiversity of the pyramids of Djoser and Lahun, especially the potential agents that cause biodeterioration that can inhabit and degrade both pyramids.

In Chapter I, the comparative microbial study of the pyramids of Djoser and Lahun was carried out through the combined use of metabarcoding methods, epifluorescence microscopy and *in vitro* cultures. Metabarcoding analysis identified 644 species of bacteria (452 genera) using the 16S rDNA gene and 204 species of fungi (146 genera) using the ITS2 region. In the isolation tests, 28 bacterial species (13 genera) and 34 species of fungi (21 genera) were identified. A total of 19 species of bacteria and 16 of fungi exclusively culture-dependent, while 92 species of bacteria and 122 of fungi were culture-independent. The most abundant SIBs were *Blastococcus aggregatus*, *B. saxobsidens* and *Blastococcus* sp. Other frequent bacteria were *Bacillus alkalitelluris*, *B. persicus*, *Planococcus salinarum* and *Planococcus* sp. The most abundant RIFs were *Knufia karalitana* and *Pseudotaeniolina globosa*,

as well as an unknown species of the Sporomiaceae family. In both pyramids, microorganisms associated with biodeterioration were detected. Microbial diversity in both pyramids was higher than expected, considering the harsh conditions of the sampling sites. It was confirmed that the best methodological approach to identify and study a complex microbial community is through the combination of microscopy and molecular identification for culture-dependent microbes and metagenomic methods for culture-independent microbes. This study is the first to simultaneously use culture-dependent and culture-independent approaches for these two pyramids and highlights the urgent need for their conservation from a microbiological perspective.

In chapter II, the morphological, physiological, and molecular characterization of a strain of the black meristematic fungus or RIF, *Pseudotaeniolina globosa* isolated in the Djoser pyramid was carried out and it was compared with an Italian strain obtained in previous works. Experiments were performed to test their preferences for temperature, salinity and pH, tolerance level to UV radiation and high temperature, as well as multilocus genotyping using five markers (ITS, nrSSU or 18S, nrLSU or 28S, BT2 and RPB2). All data confirmed the cospecificity of the two strains. However, the Egyptian was able to grow in a greater range of temperatures (4-37 °C) than the Italian (10-30 °C), at wider pH values (3.0-9.0 pH versus 4.0-6.0 pH) and at extreme levels of salinity (5 M NaCl) compared to the lowest in the Italian (0.20 M NaCl). Likewise, the Egyptian strain showed more tolerance to heat since it was able to grow after exposure to up to 85 °C for 5 min and the Italian one only up to 80 °C for 5 min, as well as to UV radiation, since the growth rate in the Egyptian was not affected for up to 9 h of exposure, unlike the Italian, which could not regrow. Based on the phylogenetic results, *P. globosa* was attributed to the group of the family Teratosphaeriaceae, order Capnodiales. Our results demonstrated that the Egyptian strain can be considered a biovariant well adapted to extreme and harsh environments that currently colonizes the Djoser pyramid of Egypt. Its potential biodeteriorating effect on such an important cultural heritage requires attention to design and seek conservation plans and solutions to limit its presence and extension in the studied area.

In Chapter III, specific molecular markers were developed to detect the presence of Actinobacteria living rapidly and inexpensively on stones (SIAb) of cultural heritage value before further analysis (e.g., metagenomics and metabarcoding). Published data from two SIB species (*Blastococcus saxobsidens* and *Geodermatophilus obscurus*) were used to extract their genomes. The search focused on the pathway for the synthesis of mycosporine-like amino acids (MAAs). These amino acids are intracellular compounds biosynthesized by the psychic acid pathway, which have been related to abiotic resistance in microorganisms. Through genomic mining, two candidate genes were obtained: a homologue of a key gene in the MAAs and the Shikimate pathways known as DAHP II (*aro*F) and another homologue of the Chorismate mutase gene (*cm*2). Both were found mainly in Actinobacteria. After calibration with five SIAb species using conventional PCR, the newly designed primers were successfully applied to environmental DNA extracted from the pyramids of Djoser and Lahun using qPCR. To our knowledge, this is the first work in which *aro*F and *cm*2 are proposed as qPCR markers to detect SIAb. The high sensitivity of qPCR to detect very low concentrations of DNA in laboratory and field samples, makes it a very useful tool for isolating SIAb species in archaeological sites. The development of new identification methods allows a greater understanding of the diversity of organisms present in outdoor monuments as it can lead to the discovery of new species or biovariants.

In the current study, combining the metabarcoding approach with the traditional isolation helped to develop a multi-step strategy to identify the diversity of microbial communities inhabit Djoser and Lahun pyramids. In the future work aim to isolate and characterize by metabarcoding detected species with unknown phylogenetic relationships. Likewise, knowing the sequences responsible for the extreme tolerance of the Egyptian strain of *P. globosa* would be of great interest, for which it would be necessary to carry out a comparison at the genomic, transcriptomic and metabolic levels using approaches based on next generation sequencing. Finally, it would be of great interest to carry out more bioinformatic analyzes to develop new molecular markers capable of detecting those groups of microorganisms found in abundance in the studied pyramids and whose identity remains unknown.

GENERAL INTRODUCTION

1. Artificial stone habitats

Stone tangible archaeological sites and monuments are invaluable proof of the prehistoric presence of man and form part of the human cultural heritage worldwide. However, they are slowly disappearing by being transformed irreversibly into sand and soil while during such natural process it may sustain life (Allsopp, Seal, & Gaylarde, 2004; Gadd, 2017). Such transformations cause a severe damage to the cultural heritage remains and may lead to a permanent loss. Beside the erosion, biodeterioration is a term to describe any damage caused by microorganisms regardless of the climatic conditions (e.g; Urzì, 2004; Trovão et al., 2019), especially for stones with cultural heritage values (Warscheid & Braams, 2000; Gorbushina et al., 2004; Scheerer, Ortega-Morales, & Gaylarde, 2009; Hermosín, Laiz Trobajo, Jurado, Miller, & Rogerio Candelera, 2018).

Stone surfaces represent a complex ecosystem, consisting of several microhabitats that vary in structure but generally with low water availability and nutrient concentration, and cause specific groups of microorganisms to proliferate. In such harsh complex ecosystem, the reported microbiome of stone surface included, fungi, lichens, cyanobacteria, actinobacteria, and other bacteria of various phylogenetic affiliations (e.g., Urzì, De Leo, de Hoog & Sterflinger, 2000; Lindahl et al., 2013; Piñar, Poyntner, Tafer, & Sterflinger, 2019).

Thus, stone or rock surfaces sustain life, its inhabitants are termed as stone-inhabiting bacteria (SIB) and rock-inhabiting fungi (RIF). SIB and RIF are generally extremophiles or extremotolerants, reported from the Antarctic, and regions of extreme dryness, and high solar irradiation (Onofri, Selbmann, Zucconi, & Pagano, 2004). Inhabiting strategy may be epilithic (i.e., found growing on the surface), chalcolithic (i.e., crevices and fissures) or endolithic (i.e., penetrate some millimeters or even centimeters into the rock pore system; Fry, 2004; Gadd, Watkinson, & Dyer, 2007). They barely surviving and rarely reproducing (i.e., live between the limit of adaptability and near-death; Friedmann & Weed, 1987).

2. Microbial diversity reported on historical monuments

2.1 Stone-inhabiting bacteria

Phototrophs SIB include Cyanobacteria, which do not require organic material for their growth, while forming biofilms and crusts on stone surfaces depending on the environmental conditions (Ortega-Morales, Narvaez-Zapata, Schmalenberger, Dousa-Lopez, & Tebbe, 2004). Cyanobacteria have been suggested to be pioneer organisms on exposed stone surfaces of buildings more often than any other organism, which facilitates the colonization and growth of subsequent heterotrophic microflora (Gaylarde & Morton, 2003). Cyanobacteria have been shown to constitute the major biomass on external surfaces of ancient stone structures in Latin America (Gaylarde, Morton, Loh, & Shirakawa, 2011), Greece (Lamprinou, Mammali, Katsifas, Pantazidou, & Karagouni, 2013) and U.S.A. (Cappitelli, Salvadori, Albanese, Villa, & Sorlini, 2012). Apart from evident aesthetic deterioration of the stone monuments they generate, these phototrophs may cause chemical and physical deterioration by the excretion of chelating agents and stone-dissolving acids (Urzì & Krumbein, 1994; May, Papida, Abdulla, Tayler, & Dewedar, 2000; Albertano, 2003).

The contribution of heterotrophic bacteria to stone deterioration was neglected for a long time, as insufficient organic nutrients were assumed to be present on stone surfaces (Scheerer et al., 2009). However, these organisms have been isolated frequently from such surfaces; and it was found that organic contaminants, such as soil, dust and dirt, are enough to support the growth of heterotrophic bacteria of oligotrophic nature (May et al., 2000).

Chemoorganotrophic bacteria utilize a wide range of nutrients and may serve other microorganisms by the breakdown of poorly degradable compounds (e.g., from atmospheric pollution), which could otherwise not be utilized (Scheerer et al., 2009). One frequent example is the species belonging to the genus *Bacillus* Cohn 1872, which have been extensively identified on stone buildings (e.g., Blazquez, Lorenzo, Flores, & Gómez-Alarcón, 2000; Heyrman & Swings, 2001; Laiz, Pinar, Lubitz, & Saiz-Jimenez, 2003; Kiel & Gaylarde, 2006).

Filamentous bacteria penetrate the substrate and excrete a wide range of enzymes and various water-soluble dark pigments (Unković et al., 2016). Laboratory experiments demonstrated their ability to utilize nitrites and nitrates and to reduce sulphates (Rivera, Ramos, Sánchez, & Serrano, 2018.) In addition, they are recognized as degraders of a wide range of different carbon and nitrogen sources (Preston et al., 2011). However, they were reported to rarely produce noteworthy amounts of organic acids and chelates in a rock decay environment (Urzì & Krumbein, 1994). Despite this, they may cause structural damage by their extensive biofilm formation and penetration of their filaments into the stone substrate (Gadd, 2017). There are several publications on the presence of actinobacteria in caves (Laiz et al., 2000; Schabereiter-Gurtner, Saiz-Jimenez, Pinar, Lubitz, & Rolleke, 2004). However, they were poorly reviewed or reported from previous studies related to cultural heritage microbiology.

The presence of chemolithoautotrophic microorganisms, such as sulfur oxidizers, nitrifying bacteria, and iron- and manganese oxidizers, depends on the availability of the specific nutrients supporting their growth (Warscheid & Braams, 2000). Gaylarde & Morton (2003) emphasized that there is little doubt that chemolithotrophic microorganisms have the potential to cause damage to stone; however, their significance to biodeterioration of outdoor stone monuments is still in question. It appears that sulfur-oxidizers and nitrifying bacteria play a more significant role in biodeterioration in humid areas, because of their sensitivity to desiccation (Warscheid & Braams, 2000).

Nitrifying bacteria have been suggested to be the most important microbial factors in the decay of sandstone (Warscheid & Braams, 2000). Sulfur-oxidizing bacteria obtain energy by the oxidation of reduced or elemental sulphur to sulfuric acid, and it may react with calcium carbonate to form calcium sulfate (gypsum), which is more soluble in water than the calcium carbonate of the parental rock (Urzì & Krumbein, 1994; Warscheid & Braams, 2000), and thus more readily leached. However, ammonia and nitrites on the stone surface are oxidized by chemolithotrophic and, partly, by heterotrophic ammonia- and nitrite-oxidizers to nitrous and nitric acid, respectively (May et al., 2000). The acids that are produced attack calcium carbonate and other minerals (Urzì &

Krumbein, 1994; Warscheid & Braams, 2000). The carbon dioxide produced can be utilized by the cells to form organic compounds, while calcium cations from the stone matrix form nitrates and nitrites, which are more soluble again than the original mineral phases and thus are leached out of the stone by rain; this process determines the characteristic discoloration and patina formation on stones (Warscheid & Braams, 2000). Many bacteria and fungi, even algae, are capable of these oxidation steps, causing damaging lesions (Caneva, Nugari, & Salvadori, 1991; Urzì & Krumbein, 1994).

2.2 Rock-inhabiting fungi

The fungal stone flora consists of filamentous fungi (ubiquitous Hyphomycetes and Coelomycetes) and meristematic micro colonial fungi (black yeasts and yeast-like fungi; May et al., 2000; Sterflinger, 2000; Gorbushina, Lyalikova, Vlasov, & Khizhnyak, 2002; Gorbushina, Diakumaku, Muller, & Krumbein, 2003). Meristematic fungi produce swollen, isodiametric cells with thick, melanin-containing cell walls; they remain metabolically active even in low nutrient conditions and have high resistance to desiccation, UV radiation and osmotic stress (Urzì et al., 2000), thus being well adapted to growth on external walls (Wollenzien, de Hoog, Krumbein, & Urzì, 1995). Hyphal penetration of materials involves swelling/deflation effects and channeling of water into the substrate; it can form cracks, fissures, and crevices, and lead to the detachment of crystals in a stone formation (Sterflinger, 2000; Urzì et al., 2000). The literature suggests that fungi are present in low numbers on the surfaces of historic stone buildings where populations of 10^2 - 10^5 cfu/g are common (Gorbushina et al., 1993; Hirsch, Eckhardt, & Palmer, 1995; Gaylarde, Gaylarde, Guiamet, de Saravia, & Videla, 2001; Ortega-Morales et al., 2016). However, this does not mean that they are unimportant; their activity may be high and erosive (Scheerer et al., 2009).

Various metabolic substances excreted by fungi are colored, leading to staining of the substrate (Tiano, 2002). The production of melanin by dematiaceous (dark pigmented mitosporic) fungi darkens the stone surface, leading to significant aesthetic alterations (Sterflinger & Krumbein, 1997). Fungi may be the most important endolithic organisms in stone formations with higher tolerance to low-water than algae and bacteria and require low nutrient

concentrations, as well as having no need for light (de los Ríos & Ascaso, 2005). Fungi were found inhabiting a variety of rock types, indicating that such environments are important fungal reservoirs (e.g., Sterflinger, 2000; Urzì et al., 2001). Fungal involvement in bio weathering of rocks and minerals is well documented (Sterflinger 2000; Fomina, Alexander, Colpaert, & Gadd, 2005; Gorbushina, Kotlova, & Sherstneva, 2008), although the majority of research focuses on fungal communities found in extreme environments, including the Arctic (Omelon, Pollard, & Ferris, 2006), Antarctic (Onofri, 1999; Ruisi, Barreca, Selbmann, Zucconi, & Onofri, 2007), hot deserts (Gorbushina, 2003) and deep subsurface environments (Gorbushina et al., 2008). Such complex terrestrial habitats can harbor a diverse range of microorganisms despite inherent harsh condition such as low water availability and lack of nutrient sources (Gorbushina et al., 2003). Some of them may even survive simulated and real space conditions (Onofri et al., 2008, 2012) and they are commonly isolated in the Mediterranean area from exposed natural rocks (Ruibal, Platas, & Bills, 2005) and monuments (De Leo, Urzì, & de Hoog, 2003; Sert, Sümbül, & Sterflinger, 2007; Egidi et al., 2014; De Leo, Antonelli, Pietrini, Ricci, & Urzì, 2019).

3. Sampling techniques from historical locations

The method used for sampling environmental microorganisms is an underestimated source of errors in determining microbial diversity (Palla & Barresi, 2017). Assessment of microorganisms associated with cultural heritage objects is particularly difficult; the damage caused in removing a representative sample must be balanced against the information gained; thus, non-destructive sampling and micro-sampling methods are preferred (Urzì & De Leo, 2001). However, these samples may not produce results that are representative of the entire extant microflora; particularly endolithic microorganisms are often overlooked by such technique (Scheerer, 2008). The limits of minimal-destructive methods, such as washing off microorganisms from rock surfaces damp cotton swaps (Mitchell & Gu, 2000; Taylor-George et al., 2005), adhesive tape (Urzì & De Leo, 2001; Gaylarde & Gaylarde, 2005) and direct contact agar (Bridson, 1969), are that only organisms on the upper

surface layers may be sampled whereas chasmolithic organisms and those that are intimately attached to the substrate may not be removed (Hirsch et al., 1995). Adhesive tape sampling allows stratigraphic examination of biofilms by consecutive sampling of the same area (Urzì & De Leo, 2001). Normally, microbial numbers decrease with rock exposed to high irradiance (Urzì, 1993; Hirsch et al., 1995). Adhesive tape sampling can also be used for fluorescent *in situ* hybridization (Urzì & De Leo, 2001; La Cono & Urzì, 2003). Destructive sampling methods include surface scraping and the removal of fragments with sterile tools (Tayler & May, 2000; de los Ríos, Galván, & Ascaso, 2004; Cappitelli et al., 2006; Ortega-Morales et al., 2016). These methods allow the assessment of more intimately attached microorganisms and those that dwell deeper in the stone.

4. Identification of the microbial communities of stone monuments

Microorganisms on or from stone may be enumerated and identified by a wide range of culture-dependent and culture-independent techniques. However, out of over 40 known prokaryotic phyla, only half have cultured representatives (Connon & Giovannoni, 2002). Culture-based techniques detect only a small fraction of the total population of an environmental sample, leading to a serious under-estimation of microbial abundance and diversity, the so-called “Great Plate Count Anomaly” (Staley & Konopka, 1985; Amann, Ludwig, & Schleifer, 1995). Most of the bacteria in culture collections were isolated on conventional, rich media, which do not mimic the oligotrophic status of natural habitats (Fry, 2004). However, conditions on outdoor stone monuments are extremely variable as well as seasonal temperature and humidity. Direct exposure to sunlight on surfaces results in higher thermal stress, desiccation, and UV radiation, and is a major factor determining the composition and functional ecology of microbial colonizers (Gaylarde, Jungblut, Gaylarde, & Neilan, 2006). A large fraction of the microbial population on outdoor stone monuments is oligo- or poikilotrophic that grow slowly but are resistant to adverse conditions, enabling them to out-grow fewer resistant microorganisms on stone surfaces; therefore, it is important to make the right choice of media to reduce the growth

of fast-growing and spreading microbes, and to prolong the incubation time for slow-growing microorganisms (Urzì, Krumbein, & Pernice, 1992).

Since the 1980s, the application of molecular ecological methods, especially those based on surveys of genes after polymerase chain reaction (PCR) amplification, allowed cultivation-independent investigations of the microbial communities in diverse environments (Rölleke, Witte, Wanner, & Lubitz, 1998).

5. Molecular methods adapted to stone cultural heritage materials

The use of DNA- and RNA-based methods in biodeterioration of cultural heritage has gained much attraction over the past decade (Sterflinger 2010; Otlewska, Adamiak, & Gutarowska, 2014). The primary use for these methods was in the detection and characterization of microorganisms in environments that are notoriously hard to culture (Otlewska et al., 2014). All the methods described below under the umbrella of nucleic acid-based techniques employ PCR-based amplification of ribosomal RNA (rRNA) or other genes, followed by fingerprinting techniques (Sanmartín, DeAraujo, & Vasanthakumar, 2018). Since the rRNA is evolutionarily well conserved and contains hypervariable regions that allow for distinguishing between closely related taxa, the bacterial and archaeal 16S rRNA gene and the 18S/28S rRNA gene/intergenic transcribed spacer (ITS) region for fungi are the gene of choice for identification. Bacterial, archaeal, and fungal ribosomal gene databases have grown to such an extent that it is now possible to identify microbial species by metagenomic approaches.

PCR-based methods and sequencing may lead to erroneous conclusions about the main organisms (Fry, 2004; Gaylarde, Shirakawa, John, Gambale, & Gaylarde, 2004). Some interfering factors, such as presence of PCR inhibitors, certain ions, metal pigments, cellulose and cellulose derivatives, or some synthetic polymers, may render results unreliable (Fry, 2004). The continues advancements in metagenomics and high-throughput sequencing have made these assays more applicable to multiple environments, especially when revealing new culture-independent microbes is required.

Until today, shotgun sequencing-based metagenomics and amplicon based next-generation sequencing methods (also known as metabarcoding) are still rarely applied.

5.1 Environmental DNA

Different methods of extracting environmental DNA (eDNA) from environmental samples differ in the degree of quality, purity, and the concentration of the isolated eDNA, especially that from arid environments, where very small amount or even frequent failure were reported (Robe, Nalin, Capellano, Vogel, & Simonet, 2003). However, common extraction protocols for bacteria or fungi were widely avoided due to main humic substances and organic residues in the soil samples that are inhibitory for PCR and restriction enzymes (Schneegurt, Dore, & Kulpa Jr, 2003). Many different protocols exist, but none were accepted and standardized for eDNA extraction from soil. However, the usage of specialized kits was previously reported, such as MO BIO Powersoil DNA isolation (Qiagen, Germany, e.g., Gourmelon et al., 2016; Baeza, Barahona, Alcaíno, & Cifuentes, 2017; Trovão et al., 2019) and FastDNA™ Spin kit for soil (MP Biomedicals, France, e.g., Bálint, Schmidt, Sharma, Thines, & Schmitt, 2014; Oh, Fong, Park, & Lim, 2016; Piñar et al., 2019).

5.2 Amplicon-based identification and metabarcoding

Metagenomic technologies have become a powerful tool for more comprehensive assessment of microbial communities in the soils of extreme environments (Cowan, Ramond, Makhalanyane, & De Maayer, 2015). It is thus of great importance that measurements of microbiomes are accurate and reliable. Amplicon-based approaches targeting variable regions of specific markers (e.g., 16S, ITS, or 18S) are widely used to describe bacterial, archaeal, and fungal diversity (Anderson & Cairney, 2004; Lindahl et al., 2013; Piñar et al., 2019).

5.2.1 Prokaryote 16S rRNA gene

The 16S rRNA gene is used in studies of microbial diversity because its sequence relatively conserved across the bacterial domain, allowing a single marker to provide taxonomic information across a very wide range of taxa, it contains conserved and variable regions (Otlewska et al., 2014). This structure

is important in its study as it allows PCR primers to be targeted at the conserved regions enabling more specificity of binding site between taxa while targeting variable regions whose greater differentiation allows for taxonomic classification (Otlewska et al., 2014).

The 16S based studies can utilize the extensive resources that were developed for its study, both in terms of analysis software (Mitra et al., 2011) and ribosomal database project (Cole et al., 2007). The greater species coverage within 16S databases compared to the number of species with fully sequenced genomes available gives 16S studies a more taxonomically diverse and species-specific reference for taxonomic identification compared to whole genome metagenomics studies. However due to the conserved nature of the 16S sequence there are issues with taxonomic resolution. Depending on the variable regions examined, as well as the taxa being evaluated, it can be impossible to differentiate taxa at or below species level (Gevers et al., 2005).

A potential issue with using 16S is the presence of multiple copies within the same genome; these copies can differ from each other by as much as 5% (Mende, Sunagawa, Zeller, & Bork, 2013), a serious issue when, by some categorizations, greater than 97% identity is sufficient to be classified as the same species (Větrovský & Baldrian, 2013). The key advantage that molecular microbial profiling has over previous culture-based methods, is the ability to detect and enumerate the unculturable bacteria (Sait, Hugenholtz, & Janssen, 2002). That is not to say that comprehensive culturing of microbes cannot complement direct sequencing methods, but they cannot be the primary methodology when trying to obtain the best approximation to the present number of taxa (Ramasamy et al., 2014).

5.2.2 Eukaryotic ITS region

In mycology, the ITS region of the nuclear ribosomal repeat unit is by far the most sequenced region for queries of systematics and taxonomy at and below the genus level. Although the ITS region is not entirely unproblematic (Bailly et al., 2007), more than 100,000 fungal ITS sequences have been deposited in the International Nucleotide Sequence Databases (INSD; Benson et al., 2018) since the early 1990s (Nilsson, Kristiansson, Ryberg, Hallenberg, & Larsson,

2008). The roughly 650 bp region is normally obtainable in a single round of Sanger DNA sequencing and of its three subregions (the spacers ITS1 and ITS2 and the 5.8S gene), two (ITS1 and ITS2) show a high rate of sequence variation and are typically species specific (Bruns, Arnold, & Hughes, 2008). A large number of ITS copies per cell upwards of 250 copies (Vilgalys & Gonzalez, 1990) made the region an appealing target for sequencing substrates where the initial amount of DNA is low, such as in environmental samples from soil. ITS rDNA is widely used to facilitate the identification of plant pathogens and ectomycorrhizal symbionts and was proposed as a potential 'barcoding' marker for fungi (Seifert, 2009; Begerow, Nilsson, Unterseher, & Maier, 2010). Jointly, these observations make a compelling case for the amplification of ITS through PCR amplification using universal primers (Kurtzman & Robnett, 1998) coupled with high-throughput sequencing (Lindahl et al., 2013) to be successfully used as a culture independent approach to study soil fungal communities.

5.2.3 Quantitative PCR

5.2.3.1 Current application

Advancements in PCR technology allow for quantification by quantitative PCR (qPCR), which enables measurement of concentration of template based on standard curves (Peirson, Butler, & Foster, 2003). The qPCR was applied on mRNA to estimate the metabolically active microorganisms in the environment through the amplification of 18S rRNA region (Michaelsen, Pinzari, Barbabietola, & Piñar, 2013) and genes in the sulfur oxidation and sulfur reduction pathways (Villa, Vasanthakumar, Mitchell, & Cappitelli, 2015). The technique was successfully applied on eDNA, amplifying and testing Beta-actin gene which was found suitable for the detection of fungal growth on historical monuments (e.g., Piñar et al., 2013; Ettenauer, Piñar, Tafer, & Sterflinger, 2014), and Cellobiohydrolase I (*cbh I*) gene and ITS, which were found useful in characterizing the microbial activity on cultural heritage monuments (Kraková, Chovanová, Puškarová, Bučková, & Pangallo, 2012).

5.2.3.2 Marker elucidation

The key to success for the qPCR technique is the application of a suitable molecular marker specific to the target group of organisms (De Gregoris, Aldred, Clare, & Burgess, 2011). However, exploring more molecular markers is not frequent in the field of cultural heritage microbiology and still in progress. A major feature for the bacterial species living in such harsh conditions, is the ability to tolerate extreme abiotic stress (Sanmartín et al., 2018).

Mycosporine-like amino acids (MAAs) are a family of intracellular compounds biosynthesized by shikimic acid pathway for the synthesis of aromatic amino acids (Bhatia et al., 2011). They have anampholyte nature and high denaturation temperature with water soluble property (Bozkurt & Kara, 2017) and found to be expressed under biotic and abiotic stresses, e.g., the protection against solar radiation (Bhatia et al., 2011) and extreme conditions (Rosic & Dove, 2011). When present within phytoplankton cells, MAAs are considered to be photochemically stable molecules acting as sunscreens against harmful UV radiation (Singh, Kumari, Rastogi, Singh, & Sinha, 2008). At times, MAAs can function as antioxidants and also as osmolytes (Wada, Sakamoto, & Matsugo, 2015).

On account of their low molecular weight, MAAs such as mycosporine-glycine, shinorine and porphyra-334 can be quickly synthesized in response to light, nutrients or temperature stress (Shick & Dunlap, 2002). High photosynthetically available radiation and UV exposure are the strongest inducing factors (Moisan et al., 2015). Exploring key genes related directly or indirectly to MAA synthase pathway would be a promising molecular marker to SIB.

6. Previous studies on artificial stone habitats of Egypt

Physical, chemical, and biological factors playing a combined role in weathering of archeological tombs. El-Anfoushi and Al-Shatby archeological tombs located in Alexandria governorate in Egypt and suffering from biodeterioration aspects were investigated using microbiological assessment. Three xerophilic fungi and

six non-xerophilic strains were isolated from Al-Shatby and El-Anfoushi archeological tombs, respectively (Afifi & Geweely, 2011).

In Tuna el-Gabel's excavations of Egypt, fungus damage was observed to passively affects various types of antiquities made of organic and inorganic materials from Ptolemaic era. Six samples taken from different archaeological objects revealed 24 fungal strains representing ten genera (Mansour & Ahmed, 2012).

Brown spots on the walls of the famous Tutankhamun's tomb were investigated for possible microbial origin. The observations indicated that the organism that created the spots is not active. After studying the microbial communities on the walls in Tutankhamun's tomb and two other nearby tombs and there were no significant differences in the numbers or types of culturable microorganisms among the three tombs sampled (Vasanthakumar, DeAraujo, Mazurek, Schilling, & Mitchell, 2013). Using pyrosequencing, no statistically significant differences in community composition and structure were observed. Fungal communities were composed primarily of *Penicillium* Link whereas the abundant bacterial taxa were members of the Firmicutes, Actinobacteria and Bacteroidetes phyla. *Penicillium chrysogenum* Thom isolated in the study produced malic acid *in vitro,* suggesting that they or other microorganisms may be responsible for the malic acid detected in the brown spots (Vasanthakumar et al., 2013).

The Seti I tomb at Luxor, the Senusret I obelisk of Al Mattaryia district, the Giza pyramid complex and related tomb, the storehouse of the National Museum of Egyptian Civilization (NMEC), the Mosque of Judge Abd El Basset (Gamaliya), the roman amphitheater of Alexandria and the Ismailia Museum of Antiquities were all found with incidences showing biodeterioration effects caused by fungi (Mohamed & Ibrahim, 2018). The Giza pyramid complex and the Museum of Ismailia Antiquities showed the lowest fungal abundances. Species from genus *Aspergillus* P. Micheli ex Haller were the most common and dominant fungal species at all archaeological sites.

7. Archaeological sites understudy

7.1 Djoser pyramid

The pyramid of Djoser (or Zoser), or step pyramid is an archeological remain in the Saqqara district, northwest of the ancient Memphis necropolis (UNESCO World Heritage Site, http://whc.unesco.org/en/list/86-002) within 29°52'10.17" N and 31°13'8.70" E in Giza governorate, southwest of the modern city of Cairo, the capital of modern Egypt. It was built during the 27th century BC for the burial of Pharaoh Djoser by Imhotep, his vizier. It is the central feature of a vast mortuary complex in an enormous courtyard surrounded by ceremonial structures and decoration; divided into two main sections; section I, namely the Djoser pyramid complex and section II the side Mastaba or tombs that start at the southern tombs entrance south of section I (Shaw, 2000). It is considered as the first Egyptian pyramid and known as the oldest building in the world built entirely of stone (Klemm & Klemm, 2001; Fig. 1). Nowadays, it is assigned as a touristic site along with its surrounding remains. Due to erosion factors, the Egyptian government started restoration in 2006 and reopened in March 2020 (https://www.reuters.com/article/us-egypt-archaeology-pyramid-idUSKBN20S1ZS).

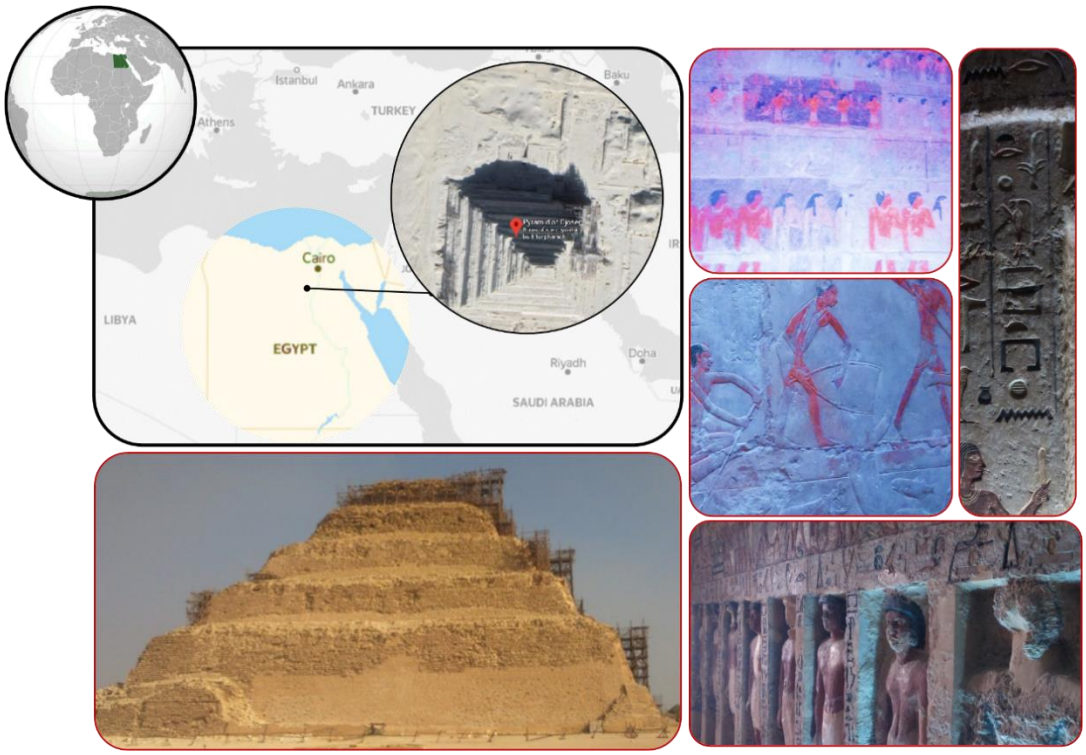

Fig. 1 Djoser pyramid mortuary complex. The approximate geographic location and some visual graphical aspects from within the complex are shown (by Samah Mohamed Rizk).

7.2 Lahun pyramid

The mud-brick Lahun pyramid of Egypt, also known as Senusret II pyramid, is located on the west bank of the Nile valley, situated within 29° 14′ 0″ N and 30° 58′ 0″ E, southern the Djoser pyramid, in Memphis necropolis. It is believed to be constructed by the pharaoh Senusret II (12th dynasty) ~ 2,000 BC and considered the first large mudbrick pyramid and was once covered by a white limestone. The outer cladding stones were locked together using dovetail inserts, some of which remain. A trench around the central core was filled with stones to act as a French Drain. The wall was encased in limestone that was decorated with niches, perhaps as a copy of Djoser's complex at Saqqara. Although still an impressive size, the pyramid is now in a ruinous condition and a natural outcrop of yellow limestone spokes around which the structure was built can be seen protruding from the rubble of the mudbrick fill in some places (Cruz-Uribe, 2010). Recently, vividly painted wooden coffins housing an anthology of pharaonic-era mummies were uncovered; the mummies are the first to be discovered beneath the sand surrounding the pyramid in the depths of the desert rock (David, 2002; Fig. 2).

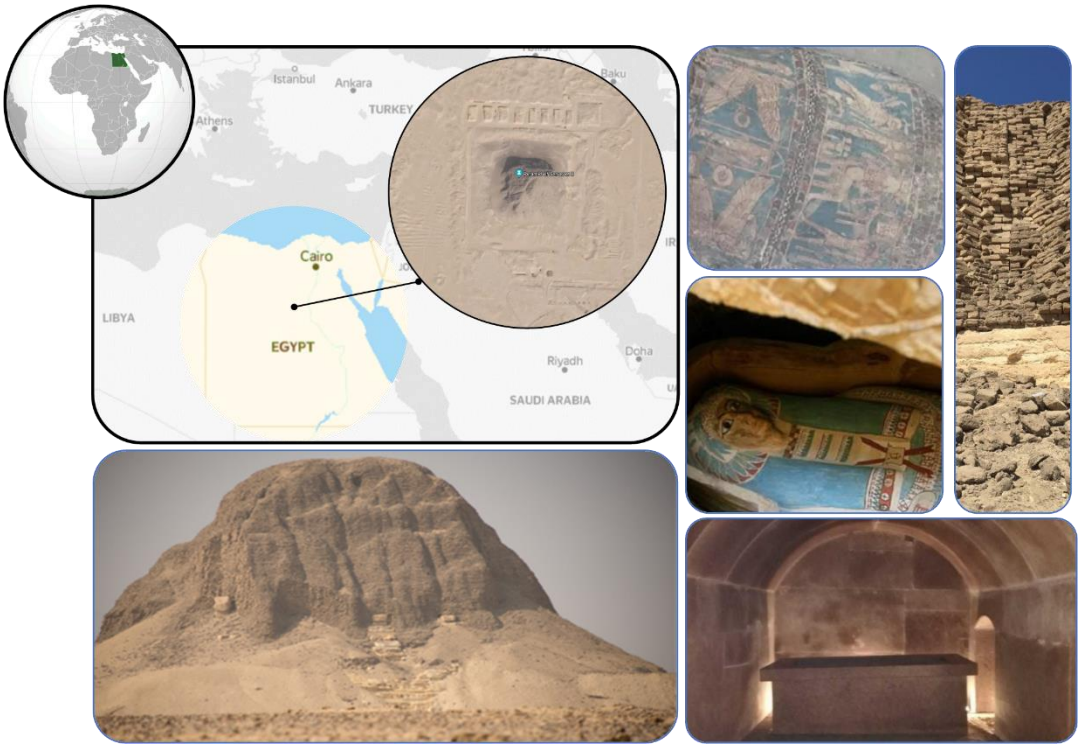

Fig. 2 Lahun pyramid mortuary complex. The approximate geographic location and some visual graphical aspects from within the complex are shown (by Samah Mohamed Rizk).

8. Objectives of the study

The general aim of this doctoral thesis was to know the microbial diversity inhabiting old artificially created microhabitats with high cultural values and exposed constantly to harsh and arid environmental conditions. Two pyramids were chosen for this study that are of the oldest and largest ones of the Memphis necropolis of ancient Egypt: Djoser and Lahun pyramids. Special attention was given to extremophilic and extremotolerant organisms as SIB and RIF. Comparative studies using metabarcoding combined with traditional isolation methods were used.

The specific objectives were the followings:

1. To know the microbial flora inhabiting Djoser and Lahun pyramids, their species richness, abundance, phylogeny, and taxonomy.
2. To study whether the identified stone-inhabiting microorganisms are potential bio-deteriorators.
3. To compare the effectiveness of the amplicon-based metabarcoding analysis and the traditional isolation methods with respect to the microbial knowledge of the studied pyramids.
4. In case that a culture-dependent microorganism of special interest was found to fully characterize it through physiological/chemical tests and by multi-locus genotyping to define its tolerance boundaries to harsh and extreme conditions and phylogenetic relationships.
5. To establish a rapid protocol applicable to cultural heritage microbiology field for SIB detection at lower cost based on genome mining and qPCR techniques.
6. To explore new SIB specific molecular markers based on a comparative genomic approach.

9. Thesis structure

The main part of this doctoral thesis is structured in three chapters. Each of them contains a study focused on different aspects of the microbial biodiversity existing on Egyptian prehistoric archeological sites (pyramids) from the pharaonic era. All the chapters are presented in the form of scientific articles followed by a general discussion and the conclusions of this thesis. A summary of each chapter is presented as follows:

Chapter I. Comparative microbial survey of Djoser and Lahun pyramids, Memphis necropolis, Egypt

In this work we aimed to detect the bio-deteriorating microbes inhabiting the Djoser and Lahun pyramids using amplicon-based metabarcoding and culture-dependent isolation methods. The species richness, their abundance, phylogeny, and taxonomy were studied (specific objectives 1, 2 and 3). Biodeterioration associated microorganisms were detected on the altered sites at both pyramids and shows their urgent need for conservation from a microbiological perspective. This is the first report of such approach applied to prehistoric tangible artificial microhabitats in Egypt.

Chapter II. A new extremotolerant biovariant of the fungus *Pseudotaeniolina globosa* isolated from Djoser pyramid, Memphis necropolis, Egypt

Based on the microbial profiling of the Djoser pyramid carried out in chapter I, a *Pseudotaeniolina globosa* isolate was reported, that is a meristematic and melanized fungus associated with monument biodeterioration with no previous taxonomical family assignment. Experiments on the Egyptian isolate were carried out to test temperature, salinity, and pH preferences, as well as stress tolerance to UV radiation and high temperature in comparison with a previous Italian isolate. In addition, a multi-locus genotyping using ITS, nrSSU or 18S, nrLSU or 28S, BT2, and RPB2 markers was made in order to know its phylogeny (specific objective 4).

Chapter III. *aro*F and *cm*2: new potential molecular markers for the detection of stone-inhabiting Actinobacteria on cultural heritage sites

By using the published data of two confirmed SIB species (*Blastococcus saxobsidens* and *Geodermatophilus obscurus)* their genomes were mined for specific molecular markers to rapidly survey the presence of SIB, specially Actinobacteria (SIAb) in cultural heritage material prior to further analysis (specific objectives 5 and 6). The search focused on the mycosporine-like amino acids (MAAs) synthesis pathway; where DAHP II (*aro*F), and Chorismate mutase gene (*cm*2) were defined as potential markers for SIAb detection. Newly designed primers were applied to eDNA extracted from Djoser and Lahun pyramidal sites using a qPCR approach. The potential of *aro*F and *cm2* as qPCR markers to detect SIAb from cultural heritage material prior to proceeding with further analysis (e.g., metagenomics and metabarcoding analyses) was evaluated.

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CHAPTER I

Comparative microbial survey of Djoser and Lahun pyramids, Memphis necropolis, Egypt

Abstract

A large percentage of the world's tangible cultural heritage is made from stone, thus deteriorating due to physical, chemical, and even biological (i.e., biodeterioration) reasons. The current study aimed to detect the bio-deteriorating microbes inhabiting two prehistoric artificial microhabitats with high cultural value in the Memphis necropolis of ancient Egypt (Djoser and Lahun pyramids) using amplicon-based metabarcoding and culture-dependent isolation methods. Sites with color alterations were sampled and examined using the epifluorescent approach before environmental DNA extraction and *in vitro* cultivation. The metabarcoding analysis identified 644 bacterial species (452 genera) using the 16S rDNA and 204 fungal species (146 genera) using ITS2. In comparison, with the isolation approach 28 bacterial species (13 genera) and 34 fungal species (21 genera) were identified. A total of 19 bacterial and 16 fungal species were exclusively culture-dependent, while 92 bacterial and 122 fungal species were culture-independent. The most abundant stone-inhabiting bacteria in the current study were *Blastococcus aggregatus*, *B. saxobsidens*, and *Blastococcus* sp., in addition to other top-represented bacteria, namely are *Bacillus alkalitelluris, B. persicus, Planococcus salinarum*, and *Planococcus* sp. The most abundant rock-inhabiting fungi were *Knufia karalitana* and *Pseudotaeniolina globosa*, besides an abundant unknown Sporomiaceae species. Biodeterioration associated microorganisms were detected on the altered sites at both pyramids. Based on previous reports, the detected stone-inhabiting microorganisms are potentially dangerous as physical and chemical erosion agents. This report is the first microbial survey using culture-dependent and -independent approaches simultaneously for those two ancient pyramids and shows their urgent need for conservation from a microbiological perspective.

1.1 Introduction

Archaeological sites and stone monuments represent an invaluable important part of the cultural heritage worldwide. These artifacts are the proof of the prehistoric presence of man and their ability to use tools for artistic purpose. A large percentage of the world's tangible cultural heritage is made from stone, and it is slowly but irreversibly disappearing by transforming the stone into sand and soil as a part of the natural recycling process, essential to sustain life on earth (Allsopp, Seal, & Gaylarde, 2004; Gadd, 2017). Thus, the deterioration of stone monuments represents a permanent loss of our cultural heritage. Biodeterioration is a term that is often used to describe any damage to any stone caused by microorganisms regardless of the climatic conditions or erosion factors (e.g., Urzì, 2004; Trovão et al., 2019). However, much attention is given to stones with cultural heritage values (Warscheid & Braams, 2000; Gorbushina et al., 2004; Scheerer, Ortega-Morales, & Gaylarde, 2009; Hermosín, Laiz Trobajo, Jurado, Miller, & Rogerio Candelera, 2018).

Despite the harsh conditions resulting from low water availability and nutrient concentration, stone surfaces represent a complex ecosystem, consisting of several microhabitats, which enable a diverse range of microorganisms to proliferate (Urzì et al., 2001). The microflora that was reported from such complex ecosystem include algae, lichens, fungi, cyanobacteria, and other bacteria of various phylogenetic affiliations (e.g., Urzì, De Leo, de Hoog, & Sterflinger, 2000; Lindahl et al., 2013; Piñar, Poyntner, Tafer, & Sterflinger, 2019). The inhabiting microorganisms may be chasmolithic, epilithic, or endolithic (Fry, 2004; Gadd, Watkinson, & Dyer, 2007). They reported from Antarctic and regions of extreme dryness and high solar irradiation (Onofri, Selbmann, Zucconi, & Pagano, 2004); live between the limit of adaptability and near-death, barely surviving and rarely reproducing (Friedmann & Weed, 1987).

Previously, in Egyptian monuments, three xerophilic fungi, namely *Aspergillus amstelodami* (L. Mangin) Thom & Church (= *Eurotium amstelodami* L. Mangin), *A. chevalieri* (L. Mangin) Thom & Church (= *E. chevalieri* L. Mangin), and *A. repens* (Corda) Sacc. (= *E. repens* Corda), and six non-xerophilic species (*Alternaria alternata* (Fr.) Keissl., *Aspergillus terreus* Thom,

A. versicolor (Vuill.) Tirab., *Cladosporium herbarum* (Pers.) Link, *Fusarium fujikuroi* Nirenberg (= *F. moniliforme* J. Sheld.), and *Penicillium chrysogenum* Thom were isolated from Al-Shatby and El-Anfoushi archeological tombs in the Alexandria governorate (Alexandria city), respectively (Afifi & Geweely, 2011).

A certain kind of fungus damage (e.g., decayed wood, contaminated bones, black spots on mud objects) was observed in Tuna el-Gabel's excavations near Al-Minya city, where different organic and inorganic materials belonging to the Ptolemaic era can be found (Mansour & Ahmed, 2012). The identified fungal isolates were *Alternaria alternata* (= *Alternaria tenuis* Nees), *Aspergillus flavus* Link, *A. humicola* Chaudhuri & Sachar, *A. niger* Tiegh, *A. terreus*, *Fusarium fujikuroi* (= *F. moniliforme*), *Bipolaris sorokiniana* Shoemaker (= *Helminthosporium sativum* Pammel, C.M. King & Bakke), *Dichotomopilus indicus* (Corda) X. Wei Wang & Samson (= *Chaetomium indicum* Corda), and *Rhizopus* Ehrenb. sp. (Mansour & Ahmed, 2012). *Aspergillus niger* and *A. terreus* were the most common and dominant fungal deteriogens followed by *A. fumigatus* Fresen, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, and *C. herbarum* in Seti I tomb (Abydos city), Senusret I obelisk (Fayoum city), Great pyramid complex (Giza city), Mosque of judge Abd El-Basset (Cairo city), and Museum of Ismailia Antiquities (Ismailia city) where fungal biodeterioration signs were revealed (e.g., black spots; Mohamed & Ibrahim, 2018).

Brown spots on the walls of the famous Tutankhamun’s tomb were investigated for possible microbial origin. Fungal communities were composed primarily of *Penicillium* Link, whereas the abundant bacterial taxa were members of the Firmicutes, Actinobacteria, and Bacteroidetes phyla (Vasanthakumar, DeAraujo, Mazurek, Schilling, & Mitchell, 2013). Actinobacteria demonstrated a great taxonomic diversity on stone surfaces and, despite the predominance of isolates of the genus *Streptomyces* Waksman & Henrici 1943, members of the genera *Geodermatophilus* Luedemann 1968, *Nocardia* Trevisan 1889, and *Rhodococcus* Zopf 1891 were also described (Groth, Vettermann, Schuetze, Schumann, & Sáiz-Jiménez, 1999). In addition to *Streptomyces* and *Nocardia*, *Micromonospora* Orskov 1923 species were also isolated from ancient stone from a tomb site in Tell Basta (Zagazig city, Egypt; Abdulla, May, Bahgat, & Deweda, 2008).

The general objective of this work was to reveal the microbial diversity inhabiting two prehistoric tangible artificial microhabitats in the Memphis necropolis of Egypt (Djoser and Lahun pyramids) with high cultural values exposed to harsh and arid environmental conditions. Precisely, the specific aims were the followings: i) to know the microbial flora (bacteria and fungi) inhabiting Djoser and Lahun pyramids, the species richness, their abundance, phylogeny, and taxonomy; ii) to check, if extremotolerant species are present; iii) to be aware of their potential biodeterioration effect of the stone-inhabiting microorganisms; iv) to compare the effectiveness of the amplicon-based metabarcoding analysis and the traditional isolation methods with respect to the microbial knowledge of the studied pyramids.

1.2 Materials and Methods

1.2.1 Archaeological sites

Geographically, the sampling area is generally characterized by Light, warm, dry sandy soil that tend to be acidic with low nutrients (Mahmoud, Alazba, Adamowski, & El-Gindy, 2015) with hyper-arid climate that is characterized by high UV exposure and day temperature, that drop drastically during the night (https://worldweather.wmo.int/). Two pyramids were chosen for this study that are of the oldest and largest ones of the Memphis necropolis of ancient Egypt. The deterioration can be observed in many parts of both pyramidal complexes in form of dark spots, coloration, and brittle rocks. The pyramid of Djoser (DP), also known as the “Step pyramid”, is an archeological remains in the Saqqara district in Memphis necropolis, located in the northern part of the Nile Valley, northwest of the city of Memphis situated at 29°52'10.17"N and 31°13'8.70"E in Giza governorate, Egypt. It was built during the 27th century BC (3rd dynasty) of limestone by Imhotep, King Djoser’s vizier (Mark, 2016). It is the central feature of a vast mortuary complex in an enormous courtyard surrounded by ceremonial structures and decoration (Shaw, 2000). It is considered the first Egyptian pyramid and known as the world’s oldest structure built entirely of stone (Hawkes, 1974).

The pyramid of Lahun (commonly spelled Al-Lāhūn; LP), also known as "Senusret II pyramid" or the mud-pyramid, is located on the west bank of the Nile valley near the opening of the Hawara Channel from the Nile Valley into the Fayum basin situated at 29°14′0″N and 30°58′0″E. It is believed to be constructed by the pharaoh Senusret II ~ 2,000 BC (12th dynasty) and considered the first large mudbrick pyramid with a yellow limestone core and was once covered entirely by white limestone (Simpson, 2001). The wall had been encased in limestone that was decorated with niches, perhaps as a copy of Djoser's complex at Saqqara; although it is still impressively large, the pyramid is now in a ruinous condition, and a natural outcrop of the yellow limestone core can be seen protruding from the rubble of the mudbrick fill in some places (Cruz-Uribe, 2010).

1.2.2 Material sampling

Six samples per pyramid were collected around the archeological sites (Table 1.1; Fig. 1.1). Soft scratches of the brittle rock formations and sandy soil from the stone structures' surfaces that showed mild color alteration (mostly grey and/or brown, and occasionally black) were collected using a sterilized scalpel and brush. The samples were put into 50 ml falcon tubes and preserved in sterile bags for further analysis. A portion of ~ 1 g was aliquoted from each sample and preserved in -20 ^{o}C for the amplicon-based metabarcoding analysis.

Table 1.1 Nature and location description of the collected samples in Djoser (DP) and Lahun (LP) pyramids; sample codes are equal to Fig. (1.1).

Code	Nature and location of samples in pyramids
DP1	Soil from the uncleared entrance of unidentified tomb near Mastaba "Khenut".
DP2	Soil beneath the false door of the Mastaba "Khenut" southwest corner.
DP3	Soil from Mastaba "Mehu" entrance.
DP4	Soil beneath the feet of four statues found in the "Heb-Sed" court.
DP5	Brittle rock precipitations of the western Massifs on the pyramid left side.
DP6	Soil from the entrance ground.
LP1	Sandy soil on the surface of the mud-rocks from the top of the pyramid.
LP2	Sandy soil on the surface of the rocks from southeastern limestone stump.
LP3	Soil from the Queen's pyramid remains.
LP4	Scratch of altered rock surface at the entrance shaft.
LP5	Scratch of altered rock surface corridor to the Burial chamber.
LP6	Scratch of altered rock surface at the ceiling of the ventilation room.

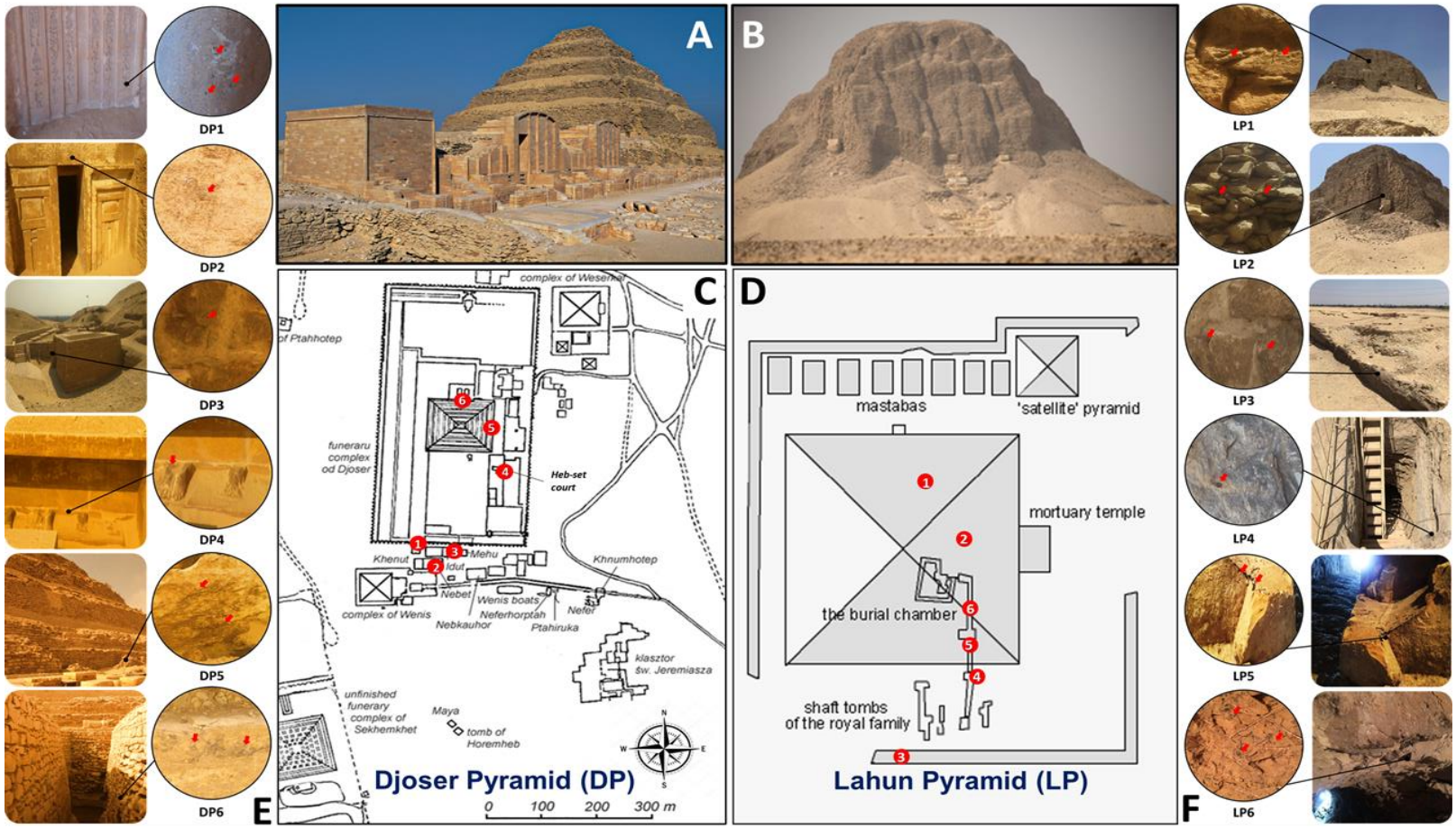

Fig. 1.1 Sampling sites information in Djoser pyramid (DP) and Lahun pyramid (LP). A, B: photographs of DP and LP respectively (by Samah Mohamed Rizk). C, D: schematic drawing of DP and LP complexes respectively showing the sampling sites by numbers (Table 1.1). E, F: sampling sites photographs showing their situation in the pyramid and the sampling spot (by Samah Mohamed Rizk).

1.2.3 Sample preparation and epifluorescence examination

Each sample was powdered in a mortar, and ~ 1 g was suspended (1:10) in a physiological solution (0.9% NaCl) with the addition of 0.001% Tween 80 and continuously agitated for 1 h at 37 °C in order to facilitate a better separation and distribution of microorganisms living in/on the rock material (Urzì et al., 2001). Epifluorescent microscope examination was performed using a drop of sample suspension prepared at the previous step and a drop of 0.1% (w/v) Acridine Orange solution. Direct observations of samples were carried out using a light epifluorescent Leica DMR microscope equipped with a mercury lamp 50 W and 450–490 nm excitation filter.

1.2.4 DNA extraction and metabarcoding analysis

Total environmental DNA (eDNA) was extracted directly from 0.25 g of each sample using the Power Soil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, United States), and quantified using a Qubit™ fluorometer (Invitrogen, Life Technologies) and the Qubit™ BR assay kit (Invitrogen, Life Technologies). Due to the low concentration of the obtained eDNA from the current material, for each pyramid samples were bulked into two sets (including three field-collected samples each) to reach the minimum concentration level required for the metabarcoding library construction (supplementary Tables 1.1 and 1.2).

For the bacteria, the V4 region rDNA of the bacterial 16S RNA gene was amplified using primers 338F (5'-ACT CCT ACG GG AGG CAG CAG-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3'; Caporaso et al., 2010). For the fungi, the ITS2 rDNA of the nuclear ribosomal RNA genes was amplified using primers ITS1F (5'-GCA TCG ATG AAG AAC GCA GC-3') and ITS2R (5'-TCC TCC GCT TAT TGA TAT GC-3'; Blaalid et al., 2013). Four independent PCR assays were performed for each bulked DNA sample under the following conditions: a 20 µl PCR reaction using TransStart FastPfu DNA Polymerase mixture contained 4 µl of 5x FastPfu Buffer, 2 µl of 2.5 mM (each) dNTPs, 0.8 µl of 5 µM Bar-PCR primer F, 0.8 µl of 5 µM primer R, 0.4 µl of FastPfu polymerase, 0.2 µl of BSA and 10 ng of genomic DNA. PCR amplification was conducted in an ABI GeneAmp® 9700 thermocycler (IET, USA) under the

following conditions: 98 °C for 3 min, 27 cycles of 10 s at 98 °C, 60 °C for 30 s, and 72 °C for 45 s, followed by 7 min at 72 °C. PCR products were examined by 2% agarose gel electrophoresis and purified using Agencourt AMPure XP beads (Beckman, USA), and were sequenced by Illumina (MiSeq, PE 2 x 300 bp mode), following Illumina instructions. Using FLASH (Magoč & Salzberg, 2011) and Trimmomatic (Bolger, Lohse, & Usadel, 2014), the pair-end reads were trimmed at any sites receiving an average quality score below 20 over a 50 bp sliding window, while reads shorter than 50 bp were discarded. The pair ends were merged with a minimum overlap length of 10 (0.2 maximum mismatch ratio). Barcodes and primer sequences at both ends were used to obtain valid sequences per sample, with 0 and 2 allowed mismatches, respectively.

The metabarcoding analysis was performed using the online Majorbio Cloud Platform (http://en.majorbio.com/). The taxonomy of each Operational Taxonomic Unit (OTU) representative sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the Silva (Release128 http://www.arb-silva.de) database for 16S rDNA and Unite (Release 7.0 http://unite.ut.ee/index.php) for ITS using a confidence threshold of 0.7. Microbial composition statistics were estimated at each taxonomic level (domain, kingdom, phylum, class, order, family, genus, and species). Mothur software (Schloss et al., 2009) was used to assess species richness and microbial diversity, while the Venn diagrams and bar-plots for the microbial composition were analyzed and illustrated using the vegan R package (https://github.com/vegandevs/vegan). The species percentage per sample shown as circus plot was elaborated using Circos-0.67 (http://circos.ca/). Phylogenetic trees were constructed by FastTree (V2.1.3 http://www.microbesonline.org/fasttree/) to reveal the genetic relationship of the species at the family level. The OTU abundance table is standardized by PICRUSt and Taxa4Fun to predict the microbial function annotations. Heatmaps were constructed using Orange software 3.24.1 (https://orange.biolab.si/). For fungi nomenclature Index Fungorum was followed (http://www.indexfungorum.org) and for bacteria the NCBI Taxonomy Browser (https://www.ncbi.nlm.nih.gov/Taxonomy/).

1.2.4 *In vitro* culture and traditional isolation of microorganisms

a) Isolation and enumeration

For the isolation and the enumeration of cultivable chemoorganotrophic microorganisms in the 12 field-collected samples, the pour plate technique (Burdass, Grainger, & Hurst, 2009) was applied by inoculating 1 ml of the sample suspension (prepared in sample preparation step) following decimal dilutions 10^{-1}:10^{-5}. The sample suspension was poured in duplicates onto Petri dishes containing two culture media: 1) BRII medium (Bunt & Rovira, 1955 modified as reported by Urzì et al., 2001) + 0.05% of the antifungal cycloheximide was utilized for chemoorganotrophic bacteria (Atlas, 2005); and 2) Geo medium (#714, List of Media for Microorganisms, DSMZ, Germany) + 0.05% cycloheximide was utilized for *Geodermatophilus* species (Atlas, 2005). The plates were then incubated at 28 °C for up to one month.

For the isolation and the enumeration of cultivable fungi, DRBC medium (Dichloran Rose Bengal Chloramphenicol agar; #CM0727, Oxoid, USA) supplemented with 100 ppm chloramphenicol to prevent bacterial growth (#SR0078, Oxoid, USA) was used. The medium was sterilized by autoclaving at 121 °C for 15 min, cooled to 50 °C, then mixed well and poured into sterile Petri dishes. Incubation was carried out at 25 °C for up to one month to allow slow-growing strains to be detected. At the end of incubation time, counts of viable microorganisms were referred to as colony-forming units *per* gram of sample (CFU/g) to detect the enumeration of the microbial community in samples per each g for bacteria and fungi (Urzì, De Leo, Bruno, & Albertano, 2010).

b) Molecular identification of microbial isolates

DNA extraction from successfully isolated bacterial strains was performed using the PureLink® Genomic DNA kit according to the kit protocol. DNA quality was checked using 1% (w/v) agarose gel electrophoresis, visualized by pre-added RedSafe® dye under UV light, and quantified using Qubit™ (Invitrogen, Life Technologies) and the Qubit™ BR assay kit (Invitrogen, Life Technologies). PCR reactions were performed using the Red Mix (BioLine, UK) kit. Each 25 µl reaction tube included 5 pmol of each primer, and 40 ng on template DNA were

added. The amplification was carried out using a Techne™ 512 thermocycler (Techne, UK). The PCR program was adjusted according to the primer pair melting temperature (Tm) as follows: the first denaturation step at 95 °C for 3 min was followed by denaturation at 95 °C for 2 s, annealing at 55 °C for 30 s, for 16S rRNA and ITS2, extension at 72 °C for 30 s. The last three steps were repeated 35 times, with the last extension step of 72 °C for 5 min. PCR products were tested using 1.5% agarose gel electrophoresis and prepared for purification using GeneJET™ PCR purification kit (Fermentas, K0702) before automated Sanger sequencing. Chromatographs were trimmed, assembled, and aligned using Geneious Prime (Kearse et al., 2012) and blasted for species identification using NCBI online Blast tool (https://www.ncbi.nlm.nih.gov/blast.cgi). Taxonomic ranking and phylogenetic relationships were retrieved from the taxonomy database (https://www.ncbi.nlm.nih.gov/taxonomy).

1.3 Results

1.3.1 Epifluorescence examination

Among the samples examined from both pyramids, direct epifluorescent microscopy observation found evidence of bacterial cells and fungal spores. The examination showed a uniform green stain in the case of bacteria, phototrophic cytoplasm showed a red autofluorescence; the nuclei of eukaryotic cells appeared green, while the cytoplasm of heterotrophs was orange (supplementary Fig. 1.1).

1.3.2 Environmental DNA extraction

The eDNA was extracted successfully for the 12 field-collected samples. The eDNA concentration ranged from 19 to 49 ng/µl with an average of 36.85±10.97 ng/µl for DP samples, and from 26 to 60 ng/µl with an average of 40.33±10.96 ng/µl for LP samples.

1.3.3 Metabarcoding analysis of the bacterial 16S rDNA

a) Raw reads information

After filtering, the four bulked samples (two sets per pyramid) recorded an average of 43,881±3,318 sequence reads, with an average nucleotide number of 18±1.5 million nt. The mean read length ranged from 413 to 422 bp; the minimum recorded sequence read length was 226 (bulk LP_S1) while the maximum was 526 bp (bulk LP_S2; supplementary Table 1.1). The total number of sequences was 232,423, with an average length of 417 bp. Using an alignment threshold of 97% similarity level, the number of classified sequences was 225,266 (96.92%), while only 7,157 sequences had 'no known relative' (3.08%).

b) Taxonomical composition

Based on the OTU identification pipeline and the Venn diagram plot, the total number of the identified OTUs in the metabarcoding samples (meta) was 940. Both pyramids shared 284 OTUs, while 104 and 552 were uniquely found in DP and LP, respectively. The OTUs identified from the two pyramids DP and LP were 388 and 836, respectively. (supplementary Fig. 1.2).

The identified OTUs were classified into higher taxonomical ranks. 644 out of the 940 OTUs were classified as species belonging to 452 genera, forming 256 families, from 142 orders, 57 classes, and 26 phyla, and all belonged to kingdom Bacteria. The portion of the major bacterial classes was visualized by abundance for each pyramid. For both pyramids, the Bacilli and Actinobacteria were the most abundant classes, however, the order of the percentage values was reversed between both pyramids. In the case of DP, the class Actinobacteria was the most abundant, followed by the Bacilli, Chloroflexia, Bacteroidia, Gammaproteobacteria, Alphaproteobacteria, and Deinococci classes, among others. On the contrary, in LP, the class Bacilli was the most abundant, followed by Actinobacteria, Bacteroidia, Gammaproteobacteria, Alphaproteobacteria, and Chloroflexia classes, among others (Fig. 1.2).

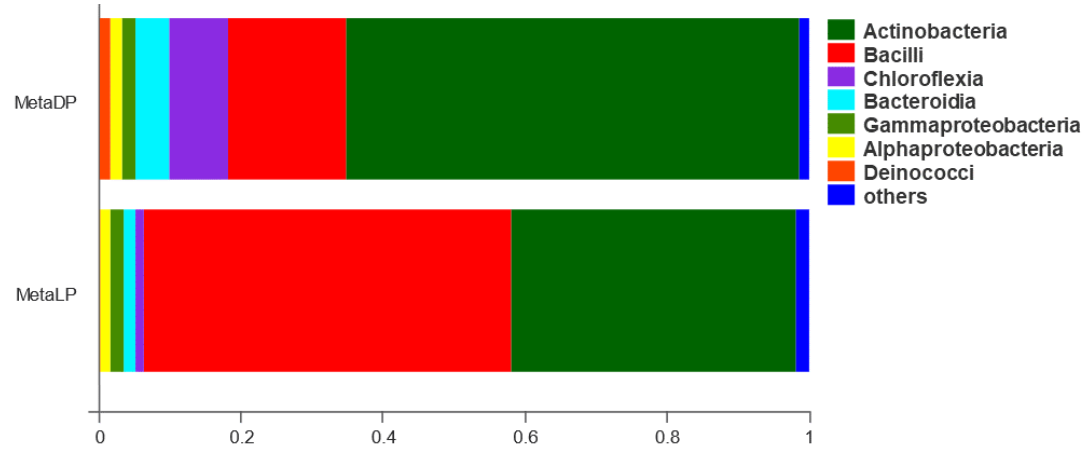

Fig. 1.2 Bacterial community composition bar-plot based on the identified OTUs for both DP and LP in the metabarcoding samples. The percentage of community abundance at the class level is shown.

When the community composition was compared between both pyramids at the family level, OTUs belonging to three major families were highly represented in both communities (i.e., at least > 1% abundance in both pyramids), namely: Planococcaceae, Micrococcaceae, and Geodermatophilaceae. Additionally, the OTUs belonging to the family Bacillaceae were highly represented in LP while weakly found (< 1%) in DP. In contrast, OTUs belonging to orders Frankiales (non-ranked) and JG30-KF-CM45 (Thermomicrobiales), and families Nocardioidaceae and Hymenobacteraceae, among others, were highly represented in DP while weakly found (< 1%) in LP (Fig. 1.3). By testing the phylogenetic relationships among the detected families, the highly represented were five bacterial classes. Class Actinobacteria was the most represented (five families), followed by Bacilli (two families), Bacteroidia, Chloroflexia, and Gammaproteobacteria (single-family; supplementary Fig. 1.3).

The top represented bacterial genera based on the metabarcoding analysis included four genera, which were in descending order *Blastococcus* Ahrens & Moll 1970, *Planococcus* Migula 1894, *Kocuria* Stackebrandt et al. 1995, and *Bacillus* Cohn 1872. According to the applied database classification, the genus *Blastococcus,* family Geodermatophilaceae, was represented by *B. saxobsidens* Urzi et al. 2004 emend. Hezbri et al. 2016, with a relative abundance ratio (rA) of 0.155 (DP) and 0.104 (LP), *B. aggregatus* Ahrens & Moll 1970 with a rA of 0.054 (DP) and 0.011 (LP), and an unclassified

Blastococcus sp., with a rA of 0.034 (DP) and 0.011 (LP). The genus *Planococcus*, family Planococcaceae was represented by *P. salinarum* Yoon et al. 2010 with a rA of 0.167 (DP) and 0.126 (LP) and an unclassified *Planococcus* sp. with a rA of 0.024 (DP) and 0.224 (LP). *Kocuria rosea* (Flugge 1886) Stackebrandt et al. 1995 represented the genus *Kocuria*, family Micrococcaceae with a rA of 0.109 and 0.234 for DP and LP, respectively. The genus *Bacillus*, family Bacillaceae was represented by *B. persicus* (Didari et al. 2013) Patel & Gupta 2020 with a rA of 0.001 (DP) and 0.129 (LP) and *B. alkalitelluris* Lee et al. 2008, with a rA of 0.008 x 10^{-1} (DP) and 0.050 (LP; Fig. 1.4).

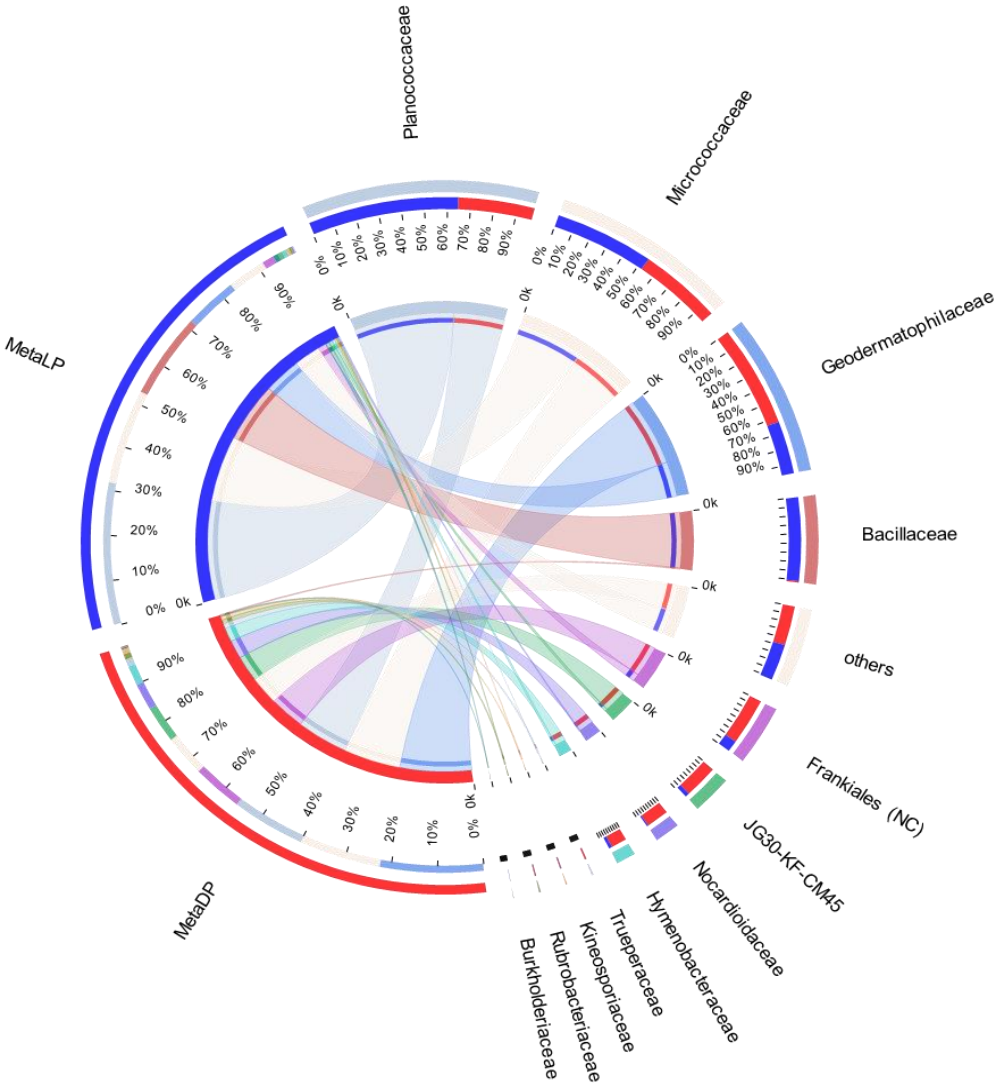

Fig. 1.3 Circus-plot for the comparative bacterial community composition based on the identified OTUs for both DP (MetaDP; red) and LP (MetaLP; blue) in the metabarcoding samples. The percentage of community abundance at the family level is shown for each pyramid. NC = not classified.

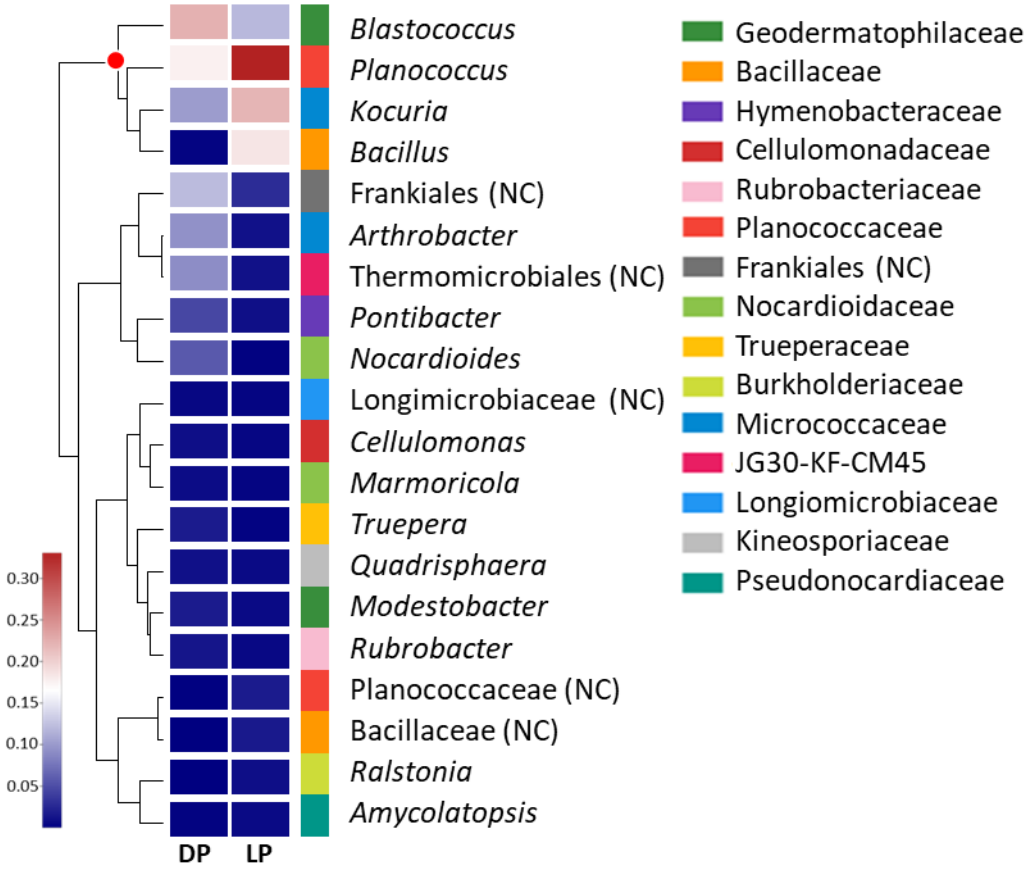

Fig. 1.4 Bacterial community heatmap for the top 20 of the most abundant genera. The relative abundance ratio is shown for the two pyramids (DP and LP) using a color scale given on the left of the figure. The family is indicated by color for each genus. The top represented genera are clustered and marked by the red dot. NC = not classified.

For each pyramid, the overall functional composition was profiled based on the identified OTUs. Both profiles were very similar; the relative abundance was the highest for bacteria characterized with genes of unknown function (S), general function (R) and amino acid transport and metabolism (E), energy production and conversion (C), among others. The latter, in which minor differences were found between both pyramids (DP > LP; supplementary Fig. 1.4).

1.3.4 Metabarcoding analysis of the fungal ITS2 region

a) Raw reads information

After filtering, the four bulked samples recorded an average of 49,483±7,111 sequence reads, with an average nucleotide number of 12.47±1.67 million nt. The mean read length ranged from 251 to 254 bp; the minimum recorded sequence read length was 140 (bulk LP_S2) while the maximum was 515 bp (bulk DP_S1; supplementary Table 1.2). Total valid sequences were 98.5K and 133.5K for DP and LP, respectively. Using an alignment threshold of 97% similarity level, the number of classified the number of classified sequences was 301,603 (99.52%), while only 1,468 sequences had 'no known relative' (0.48%).

b) Taxonomical composition

Based on the OTU identification pipeline and the Venn diagram plot, the total number of the identified OTUs in the metabarcoding samples was 306. Both pyramids shared 48 OTUs, while 43 and 215 OTUs were uniquely found in DP and LP, respectively. The OTUs identified from the two pyramids DP and LP were 91 and 263, respectively (supplementary Fig. 1.5).

The identified OTUs were classified into higher taxonomical ranks; 204 out of the 306 OTUs were classified as species, belonging to 146 genera, forming 99 families, 48 orders, 21 classes, and 6 phyla, and all belonged to kingdom Fungi. The portion of the major fungal classes was visualized by abundance for each pyramid. For both pyramids, the class Dothideomycetes was the most abundant. However, in DP, it was up to 80% compared to LP with 37%. In the case of DP, this class was followed by the Eurotiomycetes and Sordariomycetes classes, among others. In LP, the Dothideomycetes were followed by the Pezizomycetes, Sordariomycetes, Saccharomycetes, Tremellomycetes, and Eurotiomycetes classes, among others (Fig. 1.5).

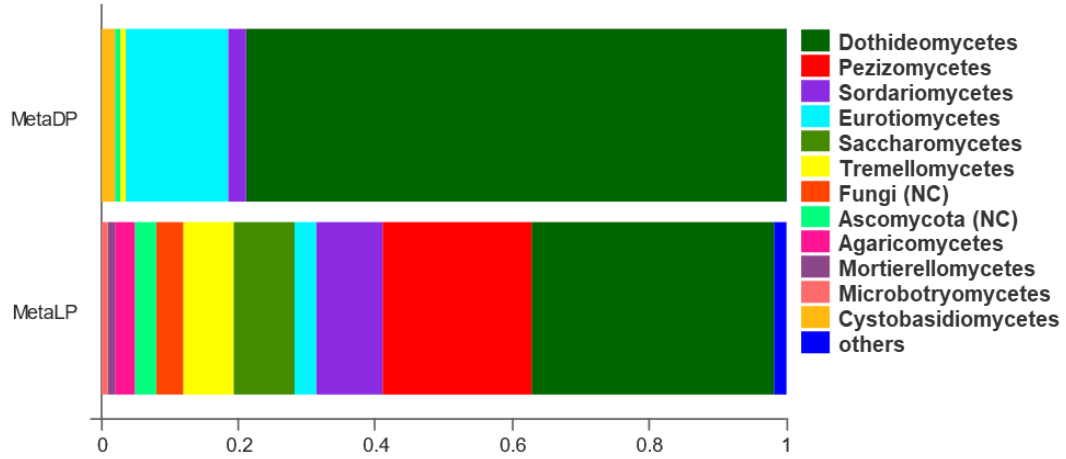

Fig. 1.5 Fungal community composition bar-plot based on the identified OTUs for both DP and LP in the metabarcoding samples. The percentage of community abundance on the class level is shown.

When the community composition was compared between both pyramids at the family level, one family was highly represented in both communities (i.e., at least > 1% abundance in both pyramids), namely: Pleosporaceae. Additionally, the families Sporomiaceae and Trichomeriaceae were highly represented in DP while weakly found (< 1%) in LP. In contrast, the families Cladosporiaceae, Mycosphaerellaceae, Pichiaceae, Sordariaceae, and Trichosporonaceae, among others, were highly represented in LP while weakly found (< 1%) in DP (Fig. 1.6). In addition, OTUs classified only to a higher level than the family that belonged to the order Pezizales, phylum Ascomycota, were highly represented in LP while weakly found (< 1%) in LP. By testing the phylogenetic relationships among the detected families, the abundant families were found to represent five classified fungal classes and two unclassified ones. The class Dothideomycetes was the most represented (four families), followed by Eurotiomycetes (two families), Sordariomycetes, Saccharomycetes, and Pezizomycetes (single-family; supplementary Fig. 1.6).

The top represented fungal genera based on the metabarcoding analysis included four genera, in descending order are *Knufia* L.J. Hutchison & Unter, *Alternaria* Nees, an unclassified member of the Sporomiaceae family and an unclassified member of Pezizales order. The genus *Knufia* was represented by *K. karalitana* Isola & Onofri, family Trichomeriaceae with a rA of 0.151, and 0.005 for DP and LP, respectively. *Alternaria chlamydospora* Mouchacca with a rA of 0.122 (DP) and 0.007 (LP) and *A. oudemansii* (E.G. Simmons) Woudenb.

& Crous with a rA of 0.301 (DP) and 0.009 (LP) represented the genus *Alternaria*, family Pleosporaceae. The unclassified species of the family Sporomiaceae showed rA values of 0.35 for DP and 0.001 for LP samples. The Pezizales unclassified species showed rA values of 0.007 x 10^{-2} for DP and 0.271 for LP samples (Fig. 1.7).

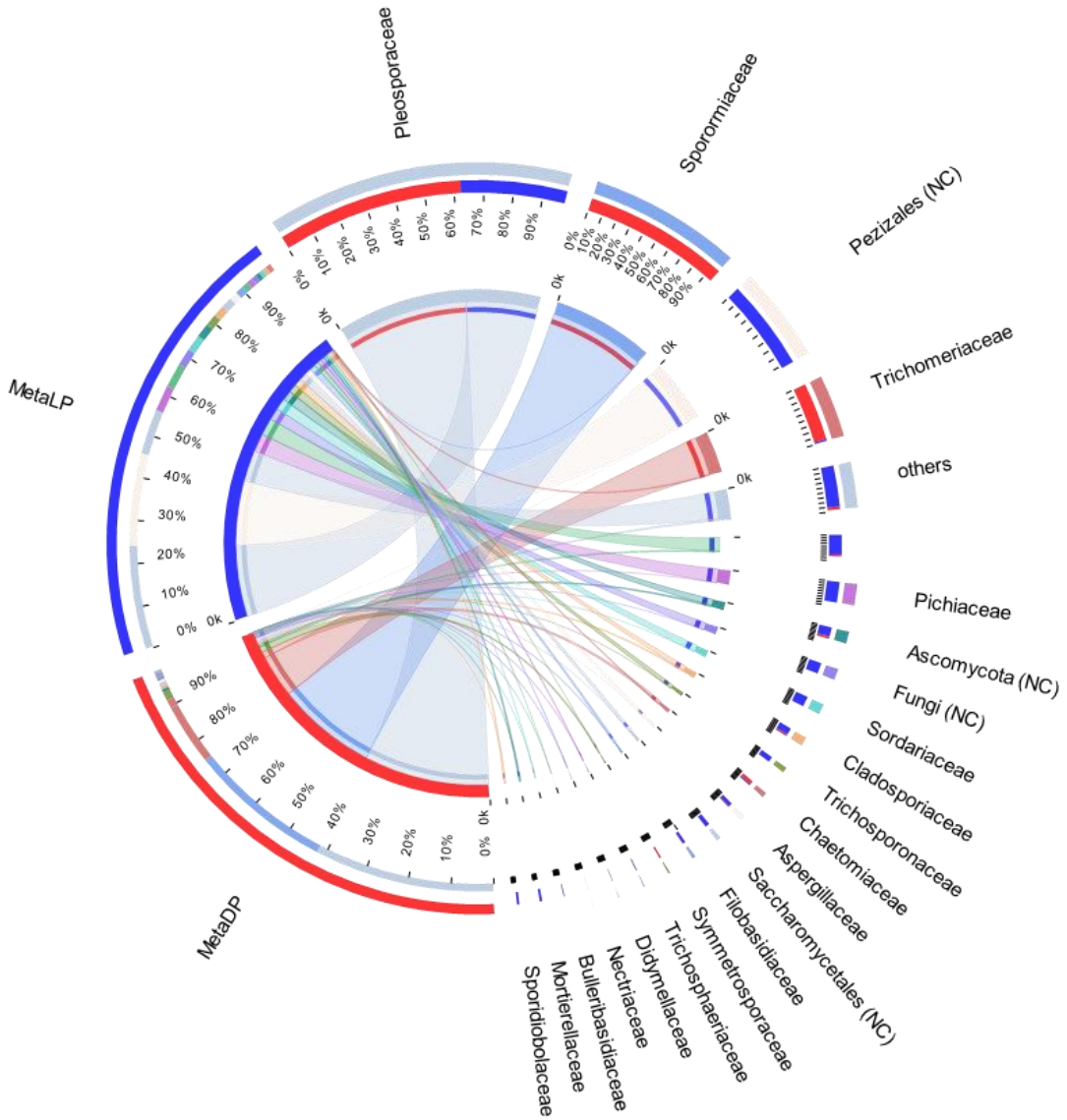

Fig. 1.6 Circus-plot for the comparative fungal community composition based on the identified OTUs for both DP (MetaDP; red) and LP (MetaLP; blue) in the metabarcoding samples. The percentage of community abundance at the family level is shown for each pyramid. NC = not classified.

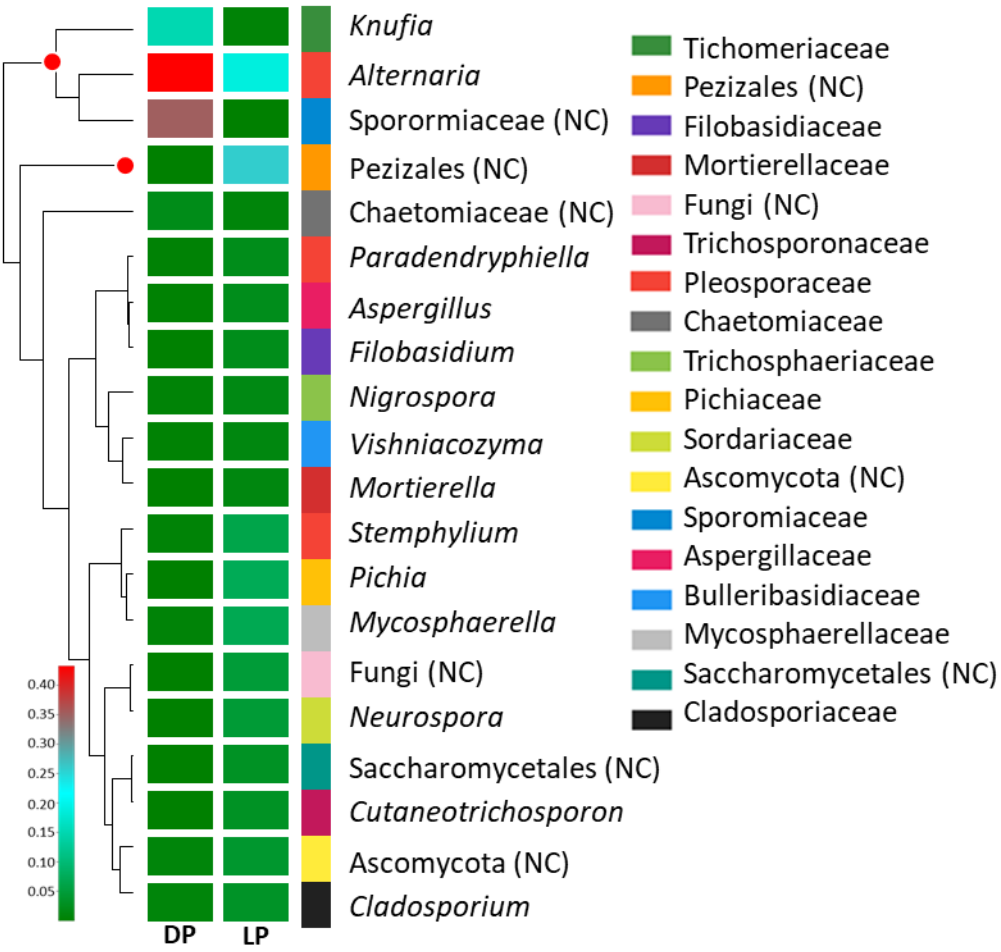

Fig. 1.7 Fungal community heatmap for the top 20 of the most abundant genera. The relative abundance ratio is shown for the two pyramids (DP and LP) using a color scale given on the left of the figure. The family is indicated by color for each genus. The top represented genera are clustered and marked by a red dot. NC = not classified.

For each pyramid, the overall functional composition was profiled based on the identified OTUs. Both profiles were very different; the relative abundance was the highest for fungi characterized as an animal pathogen, dung saprotrophs, and unknown or undefined saprotroph type. For DP, the fungi characterized as animal pathogens or dung saprotrophs were higher represented than for LP, similar to those belonging to the “Animal-Pathogen - Endophytic - Lichen Parasite - Plant-Pathogen - Wood saprotroph” group. In contrast, the fungi of unknown or undefined saprotroph type and those characterized as plant pathogens were higher presented in LP than for DP samples (supplementary Fig. 1.7).

1.3.4 *In vitro* culture analysis and traditional isolation of microorganisms

a) Enumeration of most representative colonies

Among DP samples, variable numbers of cultivable bacteria and fungi were observed. The CFU/g ranged between 1.63 and 2.33 for bacteria and 1.78 to 2.41 for fungi (DP1 was excluded since no cultivable fungi were found). The highest number of bacterial and fungal isolates was observed in sample DP6, while the lowest was found in sample DP1. Total cultivable bacteria in DP samples were 61 on BRII and 144 on Geo media, with total cultivable fungal colonies of 211. When compared to LP samples, the CFU/g ranged between 1.82 and 2.22 for bacteria and 2.00 and 2.48 for fungi. The highest number of bacterial and fungal isolates was observed in sample LP1, while the lowest was found in sample LP2. Total cultivable bacteria in LP samples were 96 on BRII and 106 on Geo media, with total cultivable fungal colonies of 278. LP showed higher number of successfully identified bacteria of 32 than DP of 20, as well as for fungi of 40 for LP to 26 for DP (Table 1.2).

Table 1.2 Enumeration of the most representative colonies isolated from Djoser (DP) and Lahun (LP) pyramids.

Samples	Cultivable bacteria (CFU/g)	Cultivable fungi (CFU/g)	Isolates count						
			a	b	c	d	e	f	g
DP1	2.22	0.00	-	33	0	-	-	3	-
DP2	2.33	1.78	24	17	12	-	1	5	2
DP3	2.30	2.41	-	40	45	-	-	4	3
DP4	2.23	2.18	-	34	30	-	-	3	3
DP5	1.63	2.23	13	-	51	1	1	3	8
DP6	2.12	2.26	24	20	73	-	-	2	10
Total DP	**-**	**-**	**61**	**144**	**211**	**1**	**2**	**20**	**26**
LP1	2.12	2.48	40	-	90	2	4	5	8
LP2	1.83	2.10	26	-	38	-	2	5	7
LP3	2.22	2.00	-	50	30	1	-	6	5
LP4	2.00	2.06	30	-	35	-	-	6	5
LP5	1.82	2.18	-	20	45	-	2	4	10
LP6	2.08	2.12	-	36	40	-	4	6	5
Total LP	**-**	**-**	**96**	**106**	**278**	**3**	**12**	**32**	**40**

a. Total cultivable bacterial colonies on BRII medium.
b. Total cultivable bacterial colonies on Geo medium.
c. Total cultivable fungal colonies on DRBC medium.
d. Count of occasionally found bacteria.
e. Count of occasionally found fungi.
f. Count of successfully identified bacteria.
g. Count of successfully identified fungi.

b) Molecular identification of microbial isolates

In general, the isolation approach surveyed 28 bacterial species (13 genera) and 34 fungal species (21 genera). In the case of DP, the bacterial 16S rDNA allowed the identification of 13 strains among the successful isolates, and the fungal ITS region rDNA enabled the identification of 15 isolates that were amplified and analyzed. At the genus level, the most frequent strains of bacteria belonged to 13 genera, namely: *Arthrobacter* Conn & Dimmick 1947, *Bacillus*, *Brevibacterium* Breed 1953, *Kocuria*, *Micrococcus* Cohn 1872, *Pseudomonas* Migula 1894 emend. Yang et al. 2013, *Streptomyces*, and *Xanthomonas* Dowson 1939 (supplementary Table 1.3).

Equally, the isolated fungi species were assigned to 13 genera, namely: *Alternaria*, *Aspergillus* P. Micheli, *Cladosporium* Link, *Curvularia* Boedijn, *Epicoccum* Link, *Fusarium* Link, *Glomerella* Spauld. & H. Schrenk, *Penicillium* Link, *Phialocephala* W.B. Kendr., and *Ulocladium* Preuss (synonym of *Alternaria*; supplementary Table 1.4). A black meristematic fungus was isolated from samples DP5 and DP6 and was assigned to the genus *Pseudotaeniolina* J.L. Crane & Schokn., which belongs to order Capnodiales *incertia sedis*.

In the case of LP, the bacterial 16S rDNA allowed the identification of 21 strains among the successful isolates, and the fungal ITS2 region enabled the identification of 26 strains that were amplified and analyzed. At the genus level, the most frequent strains of bacteria belonged to 13 genera, namely: *Agrobacterium* Conn 1942, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Clostridium* Prazmowski 1880, *Klebsiella* Trevisan 1885, *Kocuria*, *Micrococcus*, *Micromonospora* Orskov 1923, *Pseudomonas*, *Rhizobium* Frank 1889, *Streptomyces*, and *Xanthomonas* (supplementary Table 1.3).

The identified fungi were assigned to 17 genera: *Alternaria*, *Aspergillus*, *Chaetomium* Kunze, *Cladosporium*, *Curvularia*, *Epicoccum*, *Fusarium*, *Monilinia* Honey, *Mucor* Fresen, *Mycosphaerella* Johanson, *Podospora* Ces., *Puccinia* Pers., *Stachybotrys* Corda, *Stemphylium* Wallr., *Trichoderma* Persoon ex Gray, and *Ulocladium*; supplementary Table 1.4).

c) Comparative phylogenetic and taxonomic analyses

Based on the taxonomic unrooted phylogenetic tree (at genus level), the bacterial and fungal species' ranking and relationship isolated from both pyramids were defined. In the bacterial species case, the class Actinobacteria ranked first in both pyramids, while varied for Proteobacteria that ranked third in DP and second in LP, and vice versa for Firmicutes. Species belonging to genus *Clostridium* (order Clostridiales, class Firmicutes) were only found in DP, oppositely, *Agrobacterium* and *Rhizobium* species (order Rhizobiales, class Proteobacteria), *Micromonospora* sp. (order Micromonosporales, class Actinobacteria) and *Klebsiella* (order Enterobacteriales, class Proteobacteria) were only found in LP. Species of order Micrococcales (class Actinobacteria), species of genera *Bacillus* (order Bacillales, class Firmicutes), *Streptomyces* (order Streptomycetales, class Actinobacteria), and *Xanthomonas* (order Xanthomonadales, class Proteobacteria) were higher represented in DP than in LP. On the contrary, species from the genus *Pseudomonas* (order Psedomonadales, class Proteobacteria) were higher represented in LP than DP (Fig. 1.8).

In the case of the fungal species, the class Dothideomycetes ranked first, Sordariomycetes ranked second, followed by Eurotiomycetes in both pyramids. Species of genera *Glomerella* (order Glomerellales, class Sordariomycetes) and *Phialocephala* (order Ophiostomatales, class Sordariomycetes) were only found in DP. Oppositely, species of genera *Monilinia* (order Helotiales, class Leotiomycetes), *Mucor* (order Mucorales, class Mucoromycetes), and *Puccinia* (order Pucciniales, class Pucciniomycetes) were only found in LP. *Cladosporium, Mycosphaerella,* and *Pseudotaeniolina* species (order Capnodiales, class Dothideomycetes) were represented in DP more than LP, and equally for *Alternaria, Curvularia,* and *Stemphylium* species (order Pleosporales, class Dothideomycetes), but slightly lower than Capnodiales. On the contrary, *Aspergillus* species (order Eurotiales, class Eurotiomycetes) were slightly higher in LP than DP, and *Fusarium*, *Stachybotrys*, and *Trichoderma* species (order Hypocreales, class Sordariomycetes) were higher represented in LP than in DP (Fig. 1.8).

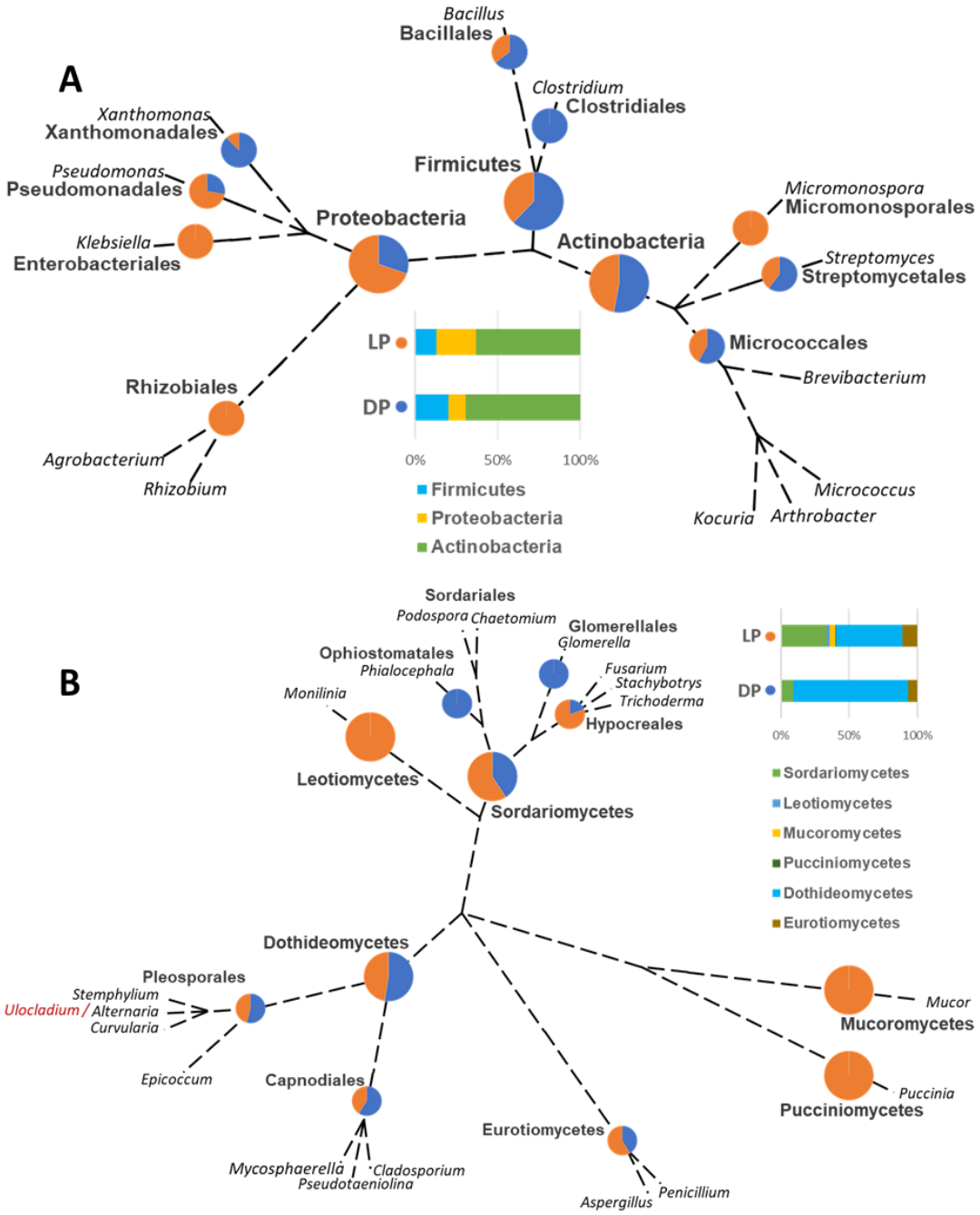

Fig. 1.8 Unrooted phylogenetic tree based on the taxonomic ranking retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/taxonomy) for the bacterial (A) and fungal (B) species isolated from the Djoser pyramid (DP) and Lahun pyramid (LP). The abundance percentage of each order and class is demonstrated as a pie chart between both pyramids at each node.

1.4 Discussion

There is no doubt that microorganisms greatly influence stone transformation and decay in a currently termed biodeterioration process. Unfortunately, such transformation is irreversible, causing significant damage to human-made stone structures, such as the monuments of cultural value and historical significance. Many reports concentrated on the microflora's diversity inhabiting such complex and harsh stone microhabitat, concluding a complex ecosystem of bacteria, fungi, algae, lichens, and protozoa (e.g., Urzì et al., 2000; Scheerer et al., 2009; Sanz et al., 2017; Pena-Poza et al., 2018). Those microorganisms are considered the second factor after erosion (i.e., physical, and chemical damage), while other studies considered them the primary cause of decay and deterioration (Warscheid & Braams, 2000; Liu, Koestler, Warscheid, Katayama & Gu, 2020). Microorganisms inhabit stone can be chalcolithic, epi- or endolithic featuring extremotolerance aspect to survive and reproduce (Rampelotto, 2013). Even though the two pyramids surveyed in the current study are similarly located in the same climatic zone and affected by very similar environmental conditions (i.e., arid climate, high exposure to UV light, and high fluctuation of temperature between day and night), the recorded microbial communities varied in composition and the dominant species at bacterial and fungal levels. However, the commonly recorded species were the key to defining the stone-inhabiting bacteria (SIB) and rock-inhabiting fungi (RIF).

A total of 19 bacterial and 16 fungal species were exclusively culture-dependent, while 92 bacterial and 122 fungal species were culture-independent. The culture-dependent identification (traditional) method is considered less informative and convenient than the culture-independent identification, as the former enabled the detection of ≤ 5% of the total microbial community (Dakal & Darora, 2012). Nevertheless, the traditional method has many benefits, the most important is the availability of the detected species and/or strains for further studies, especially when they are newly identified; however, the metabarcoding identification method has shown more advantages, considering that, in most cases, the behavior of a species is better explained in relation to the entire microbial community (Li, Zhang, He, & Yang, 2016).

The concentration of eDNA extracted from arid and hyper-arid soils, as in the case described here, is very low (Schneegurt, Dore, & Kulpa, 2003), which poses a challenge for the application of metabarcoding. In our study, bulking the samples increased the eDNA concentration and improved the 16S rDNA and ITS2 library preparation and sequencing. The metabarcoding in this study revealed microbial diversity higher than expected, considering the harsh conditions of the sampling sites (Lang Yona et al., 2018). The most common bacterial class shared between both pyramids, Actinobacteria, is considered the dominant bacteria in subterranean habitats, such as caves and tombs (Cuezva et al., 2012). Concerning the highly represented families in both pyramids, the Planococcaceae, gram-positive bacteria with no known characteristics exclusive to all members of the family, dominate. However, the known isolated species was *Planococcus salinarum*, which was previously isolated from a marine solar saltern, and is able to grow *in vitro* up to 13% w/v NaCl; (Yoon, Kang, Lee, Oh, & Oh, 2010). The species representing the family Micrococcaceae was *Kocuria rosea* known as a common soil and water species, which was found previously in extreme environments such as heavily polluted waters, deep-sea sediments, and spacecraft surfaces (Coil et al., 2016). The most abundant species belonged to the family Geodermatophilaceae, described as one of the most important stone-inhabiting Actinobacteria (Urzì et al., 2001; Sghaier et al., 2016). Two known species of the family Geodermatophilaceae, *Blastococcus aggregatus,* and *B. saxobsidens* that cause an orange coloration, were identified from both pyramids. The latter was found to resist harsh environmental conditions (e.g., UV light, ionizing radiation, desiccation, and heavy metals; Gtari et al., 2012; Montero-Calasanz et al., 2015).

Compared with the isolated culture-dependent bacterial species, the species of Geodermatophilaceae and Planococcaceae were never cultured by the currently used media. Therefore, different media compositions and protocols will be required to retrieve such isolates, especially for the two unknown species found highly represented in both pyramids (*Blastococcus* sp. and *Planococcus* sp.). Chemoorganotrophic bacteria utilize a wide range of nutrients and may serve other microorganisms by breaking down poorly

degradable compounds, which could otherwise not be utilized (Laskin, Gadd, & Sariaslani, 2009). One example is the species belonging to the genus *Bacillus*, four of which were isolated from both pyramids, and which are frequently identified on stone buildings (e.g., Kiel & Gaylarde, 2006).

An exclusive bacterium to LP belonged to the genus *Micromonospora*, which was occasionally reported from decayed stone (Ciferri, Tiano, & Mastromei, 2000), along with isolates from *Micropolyspora* Lechevalier et al. 1961 (synonym of *Nocardia* Trevisan 1889) and *Streptomyces*, were previously reported from a tomb in Tella Baste, Zagazig city in Egypt (Abdulla et al., 2008). Other isolates were best known as common environmental species of natural presence on soil (e.g., *Arthrobacter*; Eschbach, Möbitz, Rompf, & Jahn, 2003).

One of the most functional bacterial gene groups in the surveyed sites of both pyramids was the amino acid transport and metabolism; it could be very efficient in the survival in the studied sites as amino acid metabolism is associated with abiotic stress tolerance mechanisms in bacteria (Batista-Silva et al., 2019). One group is the Mycosporine-like amino acids, a family of intracellular compounds biosynthesized by the shikimic acid pathway to synthesize aromatic amino acids and are expressed under biotic, abiotic stresses (e.g., high UV exposure, Bhatia et al., 2011). Identifying related pathways would help to understand the stone-inhabiting mechanisms.

Based on ITS2 metabarcoding functional analysis, most of the detected fungi are naturally present in the soil, while some are mold, plant-pathogen species, and/or wood-inhabiting fungi (e.g., *Chaetomium globosum*). It is worth mentioning that the family Pleosporaceae (class Dothideomycetes) was one of the most abundant families in the studied pyramids, followed by family Trichomerieace (class Eurotiomycetes), which were represented by RIFs, *Alternaria chlamydospora* and *A. oudemansii* (Ruibal et al., 2009; Piñar et al., 2019), and an extremotolerant RIF, *Knufia karalitana* (Isola et al., 2016), respectively. In comparison with fungal culture-dependent isolation, seven common genera were observed at both pyramidal sites. The most remarkable isolated fungal species was a black meristematic fungus, *Pseudotaeniolina globosa* De Leo, Urzì & De Hoog, order Capnodiales *incertia sedis*, which was previously described as RIF (De Leo, Urzì, & de Hoog, 2003). The meristematic

growth is infrequent in the fungal kingdom and can be viewed as a specific response to external stress (Isola et al., 2016). RIF usually are extremotolerant microorganisms, with the ability to tolerate abiotic stress such as drought, a prolonged period of water deficiency, osmotic stress, extreme temperatures, UV radiation, and outer-space conditions (e.g., Sterflinger, 2006; Onofri et al., 2012; Zakharova et al., 2013; Sterflinger, Lopandic, Pandey, Blasi, & Kriegner, 2014; Selbmann, Zucconi, Isola, & Onofri, 2015). RIFs are known for the black coloration aspect and are very active agents causing visible alteration patterns and exfoliation of stone monuments with endolithic activity (Onofri, Zucconi, Isola, & Selbmann, 2014; De Leo, Antonelli, Pietrini, Ricci, & Urzì, 2019). By comparing our results with similar studies, several fungal genera detected in the Djoser and Lahun pyramids were previously reported from cultural heritage material. For example, *Alternaria*, *Aspergillus*, *Caldosporium, Epicoccum, Fusarium*, *Mucor*, *Penicillium* and *Trichoderma* were reported among others from storeroom objects in the Tianjin Museum, China (Liu et al., 2018), Etruscan tombs in Italy and ancient tombs of the Baekje Dynasty in Republic of Korea (Caneva, Isola, Lee, & Chung, 2020).

Based on our results, we confirm that the best methodological approach to identify and study a complex microbial community is through the combining of microscopy and molecular identification for culture-dependent microbes. Metabarcoding approaches are ideal for culture-independent microbes by the extraction of DNA and/or RNA directly from the substratum and/or biomass.

In conclusion, upon the survey of two of the oldest and largest pyramids in Memphis necropolis of ancient Egypt, Djoser and Lahun pyramids, both are inhabited by potential biodeterioration agents, some known for their ability to transform the stone surface and rock formation, and potentially as dangerous as physical and chemical erosions. The identified SIB previously related to biodeterioration were *Blastococcus aggregatus*, *B. saxobsidens* and *Blastococcus* sp., while *Bacillus alkalitelluris*, *B. persicus, Planococcus salinarum*, and *Planococcus* sp. will need further investigation to examine their biodeterioration effect. The surveyed RIF in the current study were *Knufia karalitana* and *Pseudotaeniolina globosa*, in addition to a species belonging to the family Sporomiaceae, which will need further isolation and identification.

1.5 References

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1.6 Supplementary Material

Supplementary Table 1.1 Raw reads statistics obtained from Illumina run of the 16S rDNA.

DNA Bulk Code	Sequence reads	Base number	Mean length	Min. length	Max. length
DP_S1	42,470	17,527,690	413	401	441
DP_S2	41,166	17,077,794	415	339	439
LP_S1	43,189	18,179,685	421	226	471
LP_S2	48,697	20,536,504	422	261	526

Supplementary Table 1.2 Raw reads statistics obtained from Illumina run of the ITS2 region.

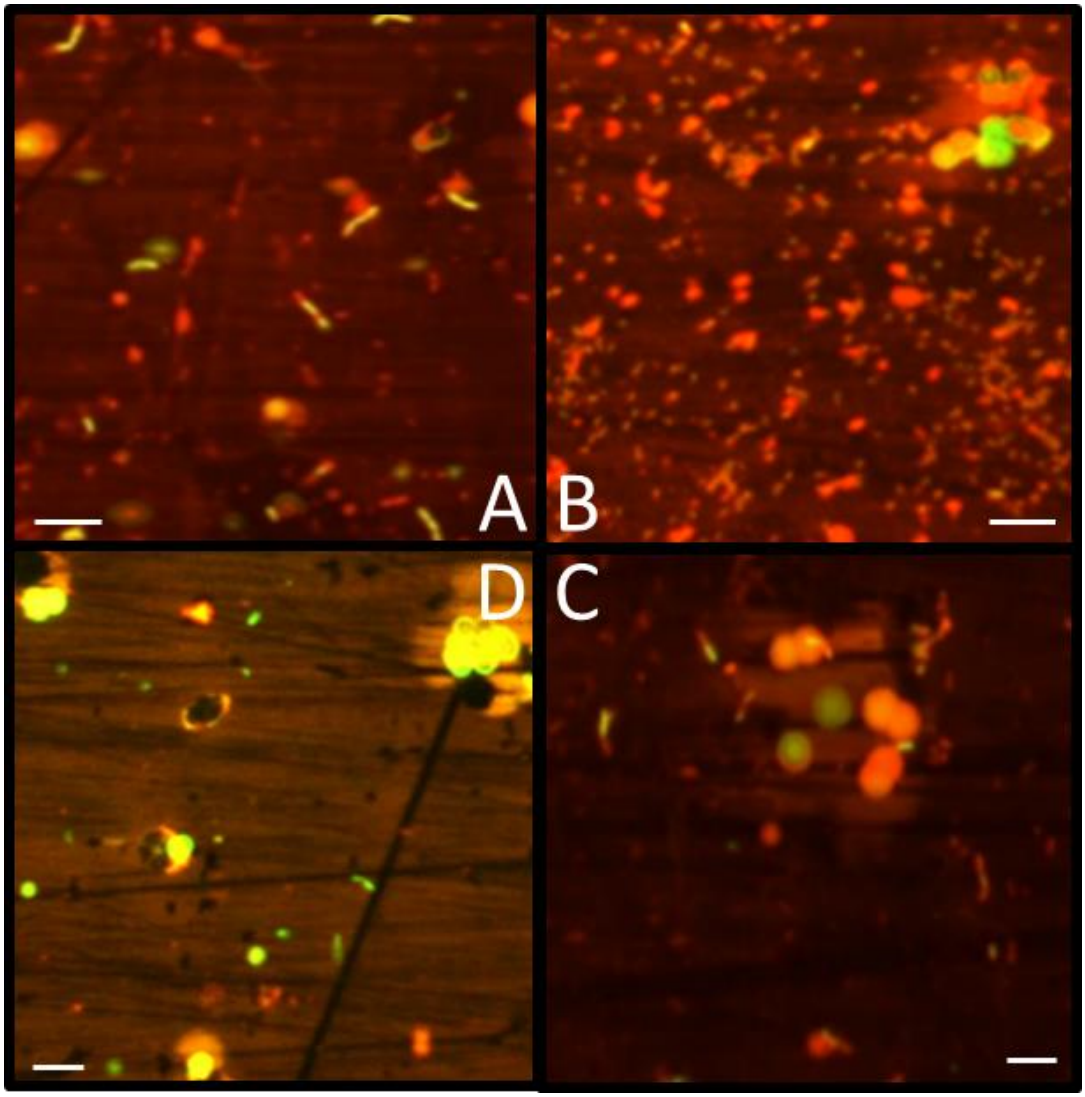
DNA Bulk Code	Sequence reads	Base number	Mean length	Min. length	Max. length
DP_S1	54,512	13,662,045	251	166	331
DP_S2	44,455	11,295,272	254	187	515
LP_S1	71,434	17,006,153	238	147	343
LP_S2	62,872	16,523,855	263	140	504

Supplementary Table 1.3 Molecular identification based 16S rDNA sequencing for the successful isolates and its percentages from Djoser (DP) and Lahun (LP) pyramids samples. Shaded rows are unique taxa isolated from one of the two pyramids.

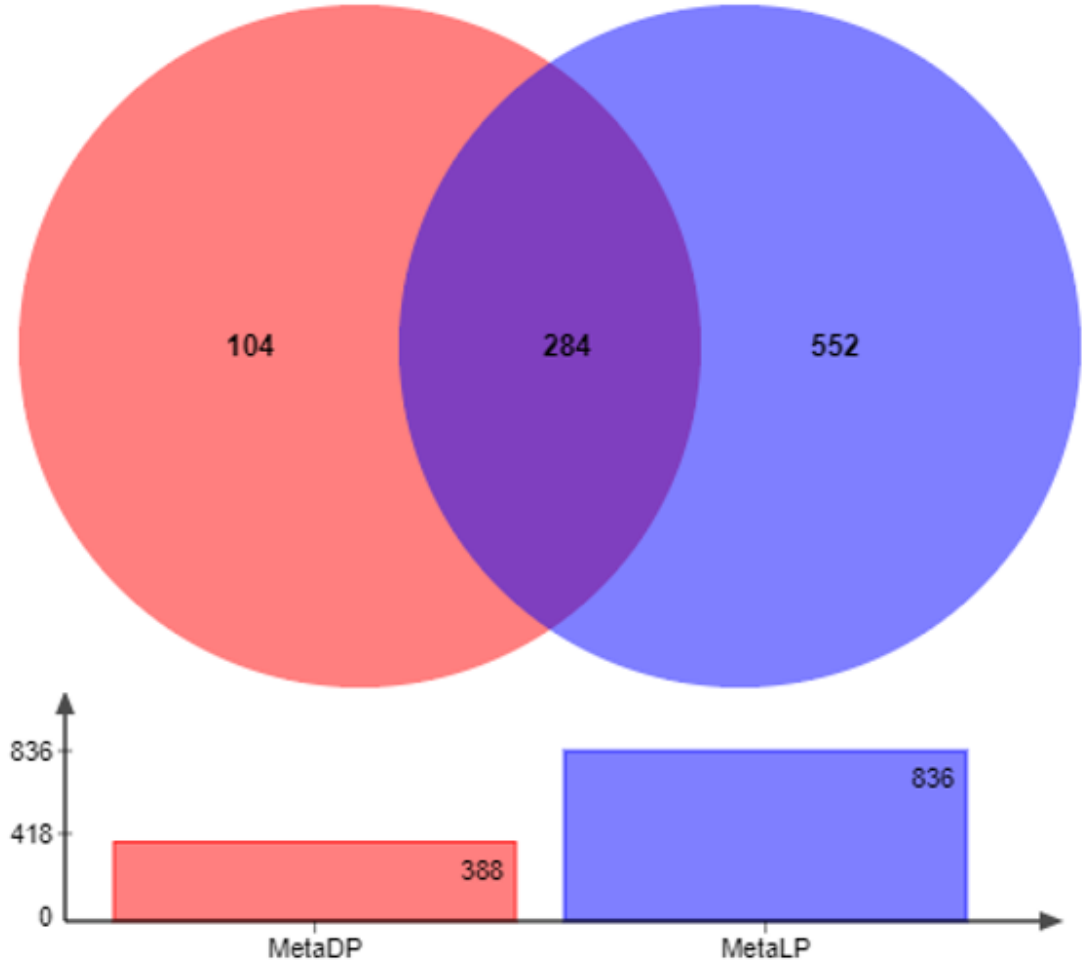
Bacteria	Isolates %											
	Djoser Pyramid						Lahun Pyramid					
	DP1	DP2	DP3	DP4	DP5	DP6	LP1	LP2	LP3	LP4	LP5	LP6
Agrobacterium tumefaciens (Smith and Townsend 1907) Conn 1942	-	-	-	-	-	-	25	-	-	-	-	-
Arthrobacter agilis (Ali-Cohen 1889) Koch et al. 1995	-	-	-	-	-	-	-	30.8	-	-	-	-
Arthrobacter parietis Heyrman et al. 2005	-	-	-	58.8	-	-	-	-	-	-	-	16.7
Bacillus flexus (ex Batchelor 1919) Priest et al. 1989	-	-	-	-	-	-	12.5	-	-	-	-	-
Bacillus pocheonensis Ten et al. 2007	-	-	-	-	-	-	-	-	-	16.7	30	-
Bacillus safensis Satomi et al. 2006	-	19.5	-	-	40.5	-	-	-	-	-	-	-
Bacillus subtilis (Ehrenberg 1835) Cohn 1872	-	-	35	26.5	-	-	-	7.7	-	-	-	-
Brevibacterium iodinum (ex Davis 1939) Collins et al. 1981	-	-	-	-	10.8	-	-	-	-	-	-	-
Brevibacterium Breed 1953 **sp.**	-	-	-	-	-	-	-	34.6	-	-	-	-
Clostridium Prazmowski 1880 **sp.**	-	-	-	-	-	-	-	7.7	-	-	-	-
Klebsiella oxytoca (Flugge 1886) Lautrop 1956	-	-	-	-	-	-	12.5	-	-	40		
Kocuria sediminis Bala et al. 2012	21	-	27.5	-	-	-	-	19.2	14	-	-	5.6
Kocuria Stackebrandt et al. 1995 **sp.**	-	12.2	-	-	-	-	-	-	-	30	-	-
Kocuria turfanensis Zhou et al. 2008 emend. Camacho et al. 2017	52	29.3	-	-	48.9	-						
Micrococcus luteus (Schroeter 1872) Cohn 1872	-	-	17.5	-	-	-	22.5	-	20	6.7	-	-
Micromonospora Orskov 1923 **sp.**							-	-	22	-	35	22.2
Pseudomonas fluorescens Migula 1895							-	-	-	-	15	11.1
Pseudomonas oryzihabitans Kodama et al. 1985	-	-	-	14.7	-	-						
Pseudomonas Migula 1894 emend. Yang et al. 2013 **sp.**	-	-	-	-	-	-	-	-	12	-	-	-
Rhizobium Frank 1889 **sp.**	-	-	-	-	-	-	20	-	-	-	-	-
Streptomyces flavoviridis (ex Preobrazhenskaya et al.) Preobrazhenskaya 1986	-	-	-	-	-	-	-	-	12	-	-	-
Streptomyces griseoflavus (Krainsky 1914) Waksman and Henrici 1948	-	22	-	-	-	53.8	-	-	-	-	-	-
Streptomyces griseus (Krainsky 1914) Waksman & Henrici 1948	-	-	-	-	-	-	-	-	16	-	-	-
Streptomyces heliomycini (ex Braznikova et al. 1958) Preobrazhenskaya 1986	-	17.1	-	-	-	-	-	-	-	-	20	11.1
Streptomyces lavendulocolor (Kuchaeva et al. 1961) Pridham 1970	-	-	-	-	-	-	-	-	-	-	-	33.3
Streptomyces marokkonensis Bouizgarne et al. 2009	-	-	-	-	-	46.2	-	-	-	-	-	-
Xanthomonas campestris (Pammel 1895) Dowson 1939	27	-	20	-	-	-	-	-	-	-	-	-
Xanthomonas Dowson 1939 **sp.**	-	-	-	-	-	-	-	-	-	6.7	-	-
Un-identified	-	-	-	-	-	-	7.5	-	4	-	-	-

Supplementary Table 1.4 Molecular identification based on ITS2 region sequencing for the successful isolates and its percentages from Djoser (DP) and Lahun (LP) pyramids samples. Shaded rows are unique taxa isolated from one of the two pyramids.

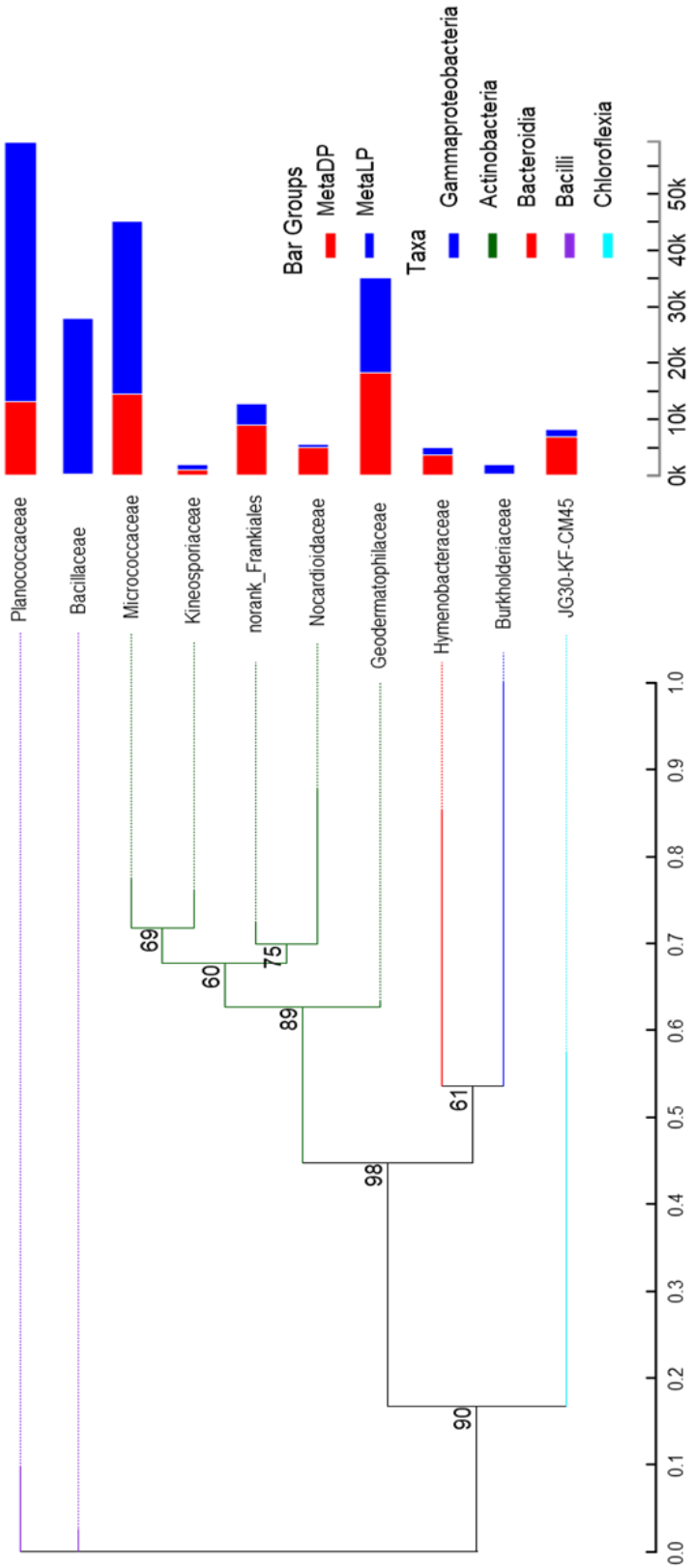
Fungi	Isolate %											
	Djoser Pyramid						Lahun Pyramid					
	DP1	DP2	DP3	DP4	DP5	DP6	LP1	LP2	LP3	LP4	LP5	LP6
***Alternaria alternata* (Fr.) Keissl.**	-	-	-	-	-	-	16.7	-	-	-	-	-
Alternaria iridiaustralis E. G. Simmons, Alcorn & C. F. Hill	-	-	-	-	-	-	-	-	-	-	6.7	-
Alternaria obovoidea (E.G. Simmons) Woudenb. & Crous	-	-	-	30	21.6	9.6	-	13.2	20	-	-	-
Alternaria Nees **sp.**	-	-	-	-	-	5.5	-	-	-	-	-	-
Aspergillus flavus Link	-	-	-	-	-	11	-	-	-	-	-	-
Aspergillus niger Tiegh.	-	-	-	-	9.8	-	-	-	-	-	6.7	-
Aspergillus sydowii (Bainier & Sartory) Thom & Church	-	-	-	-	-	-	20	-	-	-	-	-
Aspergillus terreus Thom	-	-	-	-	-	-	-	10.5	-	-	11.1	-
Chaetomium globosum Kunze	-	-	-	-	-	-	12.2	-	-	17.1	-	12.5
Cladosporium halotolerans Zalar, de Hoog & Gunde-Cim.	-	-	-	-	-	-	-	-	-	-	6.7	20
***Cladosporium* Link sp.**	-	-	20	26.7	13.7	23.4	13.4	-	-	-	-	-
Cladosporium velox Zalar, de Hoog & Gunde-Cim.	-	-	-	-	-	-	-	23.7	-	-	-	-
Cladosporium fulvum Cooke	-	-	-	-	-	-	-	-	-	8.6	-	-
Curvularia lunata (Wakker) Boedijn	-	-	-	-	-	9.6	-	-	10	43.8	-	-
Epicoccum nigrum Link	-	83.3	-	-	7.8	-	-	15.8	16.7	-	-	27.5
Epicoccum Link **sp.**	-	-	-	-	10.4	5.5	-	-	-	-	-	-
Fusarium equiseti (Corda) Sacc.	-	-	-	-	-	8.2	-	-	-	-	-	-
Fusarium oxysporum Schltdl.	-	-	-	-	-	-	5.	-	-	-	-	12.5
Fusarium Link **sp.**	-	-	-	-	13.7	-	-	-	-	10.5	8.9	-
Glomerella cingulata (G. F. Atk.) Spauld. & H. Schrenk	-	-	-	-	-	6.8	-	-	-	-	-	-
Monilinia Honey **sp.**	-	-	-	-	-	-	-	7.9	-	-	-	-
Mucor ambiguous Vuill.	-	-	-	-	-	-	-	5.3	-	14.3	-	-
Mycosphaerella Johanson **sp.**	-	-	-	-	-	-	7.77	-	-	-	-	-
Mycosphaerella tassiana (De Not.) Johanson	-	-	-	-	-	-	-	-	23.3	-	8.9	-
Penicillium Link **sp.**	-	-	-	-	13.4	-	-	-	-	-	-	-
Phialocephala fluminis Shearer, J. L. Crane & M. A. Mill.	-	16.6	-	-	-	-	-	-	-	-	-	-
Podospora anserina (Rabenh.) Niessl	-	-	-	-	-	-	-	-	-	-	20	-
Pseudotaeniolina globosa De Leo, Urzi & De Hoog 2003	-	-	57.8	-	-	20.5	-	-	-	-	-	-
Puccinia graminis Pers.	-	-	-	-	-	-	-	-	-	-	2.2	-
Stachybotrys chlorohalonatus B. Andersen & Thrane	-	-	-	-	-	-	-	-	30	-	6.7	10
Stemphylium Wallr. **sp.**	-	-	-	-	-	-	10	18.4	-	-	-	-
Trichoderma Pers. **sp.**	-	-	-	-	-	-	6.7	-	-	-	-	-
Ulocladium botrytis Preuss	-	-	-	-	-	-	-	-	-	-	15.6	-
Ulocladium Preuss **sp.**	-	-	22.2	43.3	9.5	-	-	-	-	5.71	-	-
Un-identified	-	-	-	-	-	-	7.8	5.3	-	-	6.7	17.5



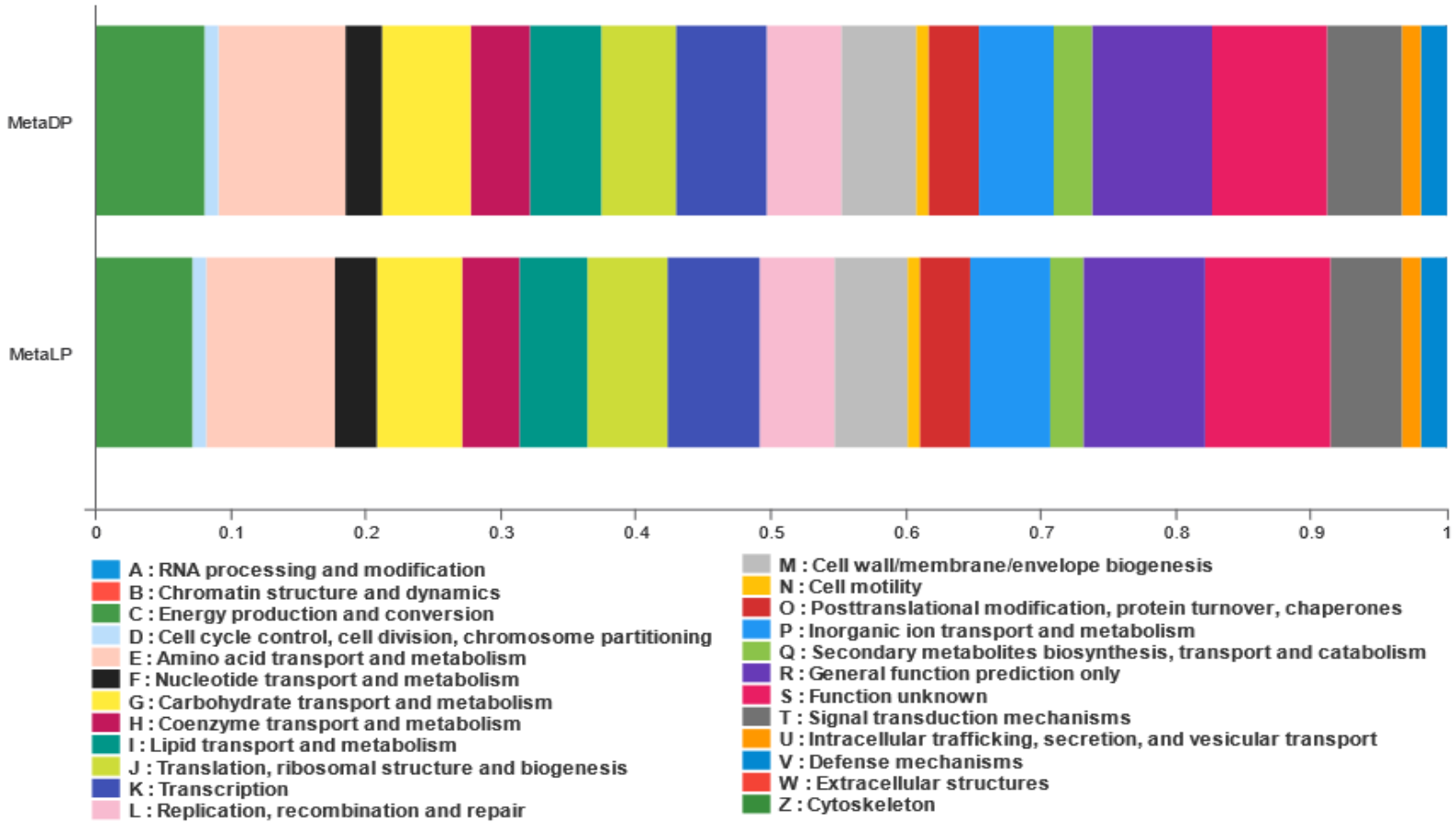
Supplementary Fig. 1.1 Epifluorescent microscopic observation of some samples stained with Acridine Orange on a slide. Phototrophic cytoplasm shows a red autofluorescence; the nuclei of eukaryotic cells appeared green, while the cytoplasm of heterotrophs was orange. A: mixed microbial community mainly prokaryotic with some eukaryotic presence from DP2. B: from DP5 site showing mixed microbial community mainly composed of phototrophs with a denser eukaryotic presence of green color in the upper corner. C: sample from LP2 site showing dominant presence of eukaryotic microorganisms with low number of phototrophs color signals. D: examined sample from LP4 site showing mixed microbial community of bacteria in red clumps and fungal or algae cells in green (bar = 10 μm).



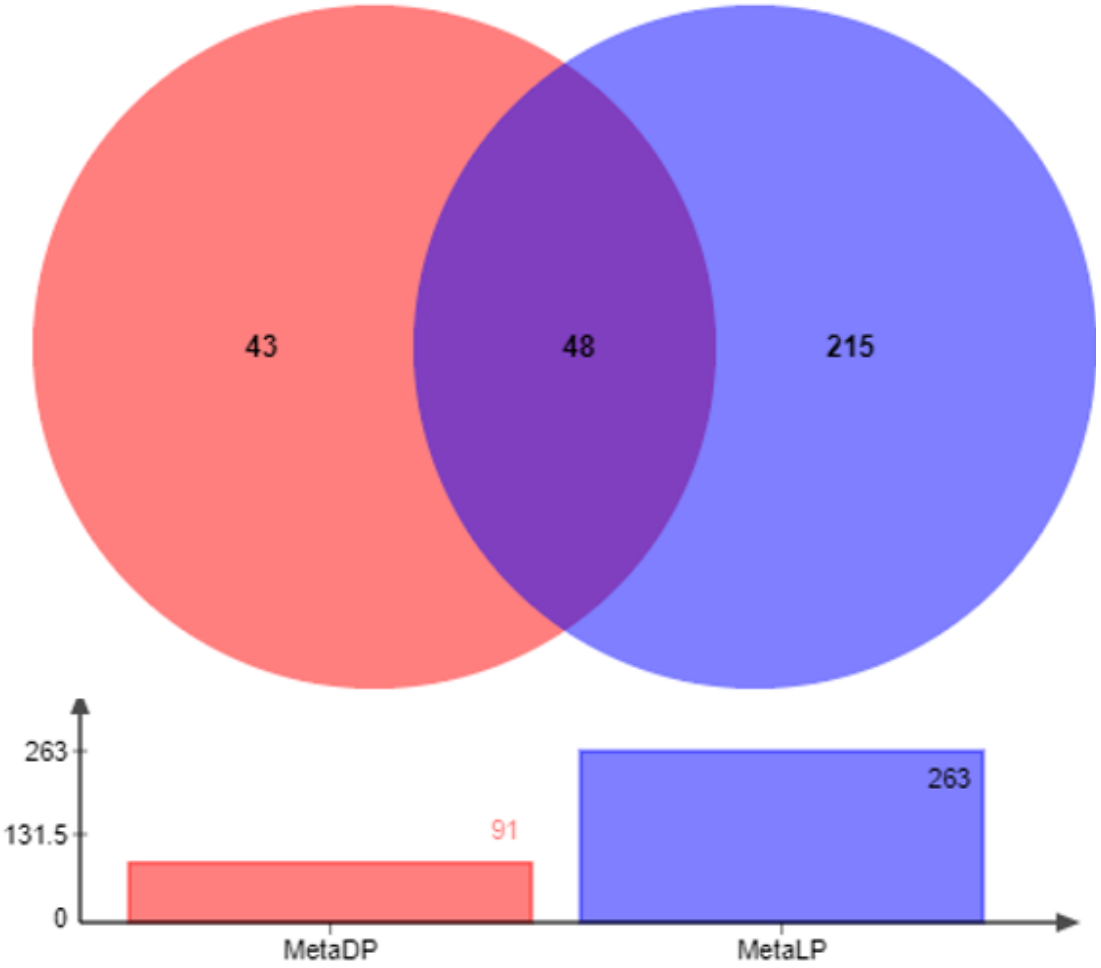
Supplementary Fig. 1.2 Histogram and Venn diagram plot for the identified OTUs based on bacterial 16S rDNA metabarcoding analysis from the two pyramids DP (red) and LP (blue).



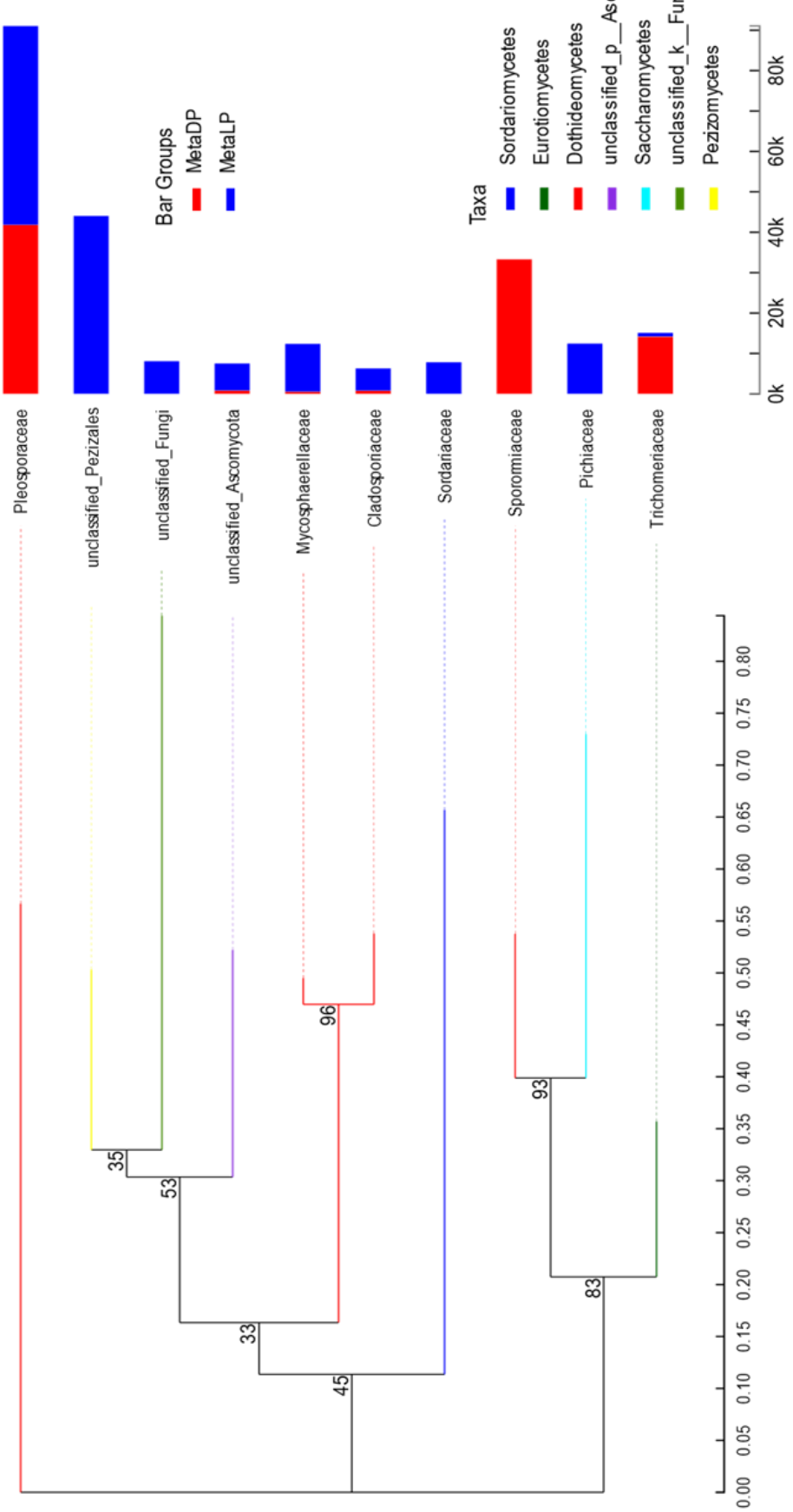
Supplementary Fig. 1.3 16S rDNA phylogenetic tree of the highly abundant bacteria families found shared between the DP and LP samples. Each family on the tree is colored by class; for each family, the total number of sequences is presented for each pyramid (DP: red and LP: blue).



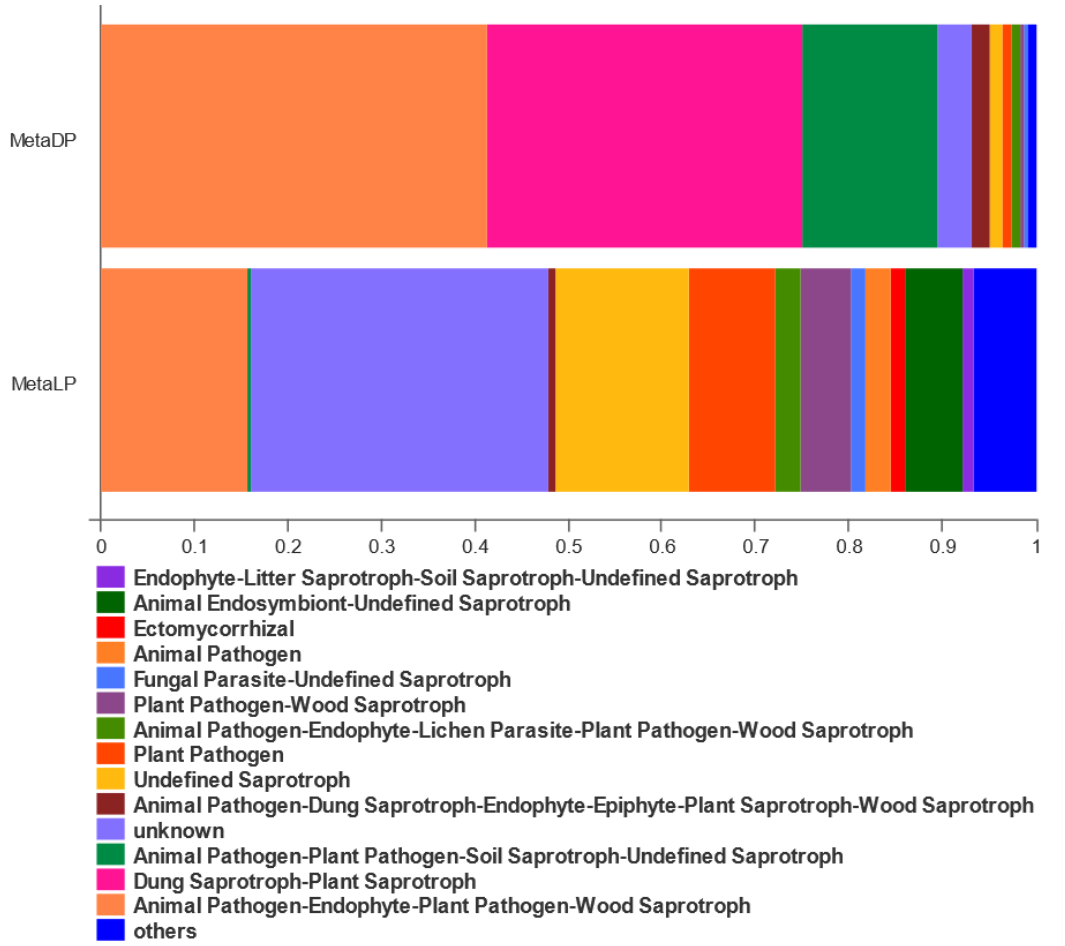
Supplementary Fig. 1.4 Functional profile of the bacterial community of both pyramids, DP and LP, based on the identified OTUs in the metabarcoding samples. Each functional group is colored and lettered alphabetically from A to Z**.** The relative abundance of each function is indicated.



Supplementary Fig. 1.5 Histogram and Venn diagram plot for the identified OTUs based on the ITS2 region metabarcoding analysis from the two pyramids DP (red) and LP (Blue).



Supplementary Fig. 1.6 ITS2 region phylogenetic tree of the highly abundant fungal families found shared between the DP and LP samples. Each family on the tree is colored by class; for each family, the total number of sequences is presented for each pyramid (DP: red and LP: blue).



Supplementary Fig. 1.7 Functional profile of the fungal community of both pyramids, DP and LP, based on the identified OTUs in the metabarcoding samples. Each functional group is colored. The relative abundance of each function is indicated.

CHAPTER II

A new extremotolerant biovariant of the fungus *Pseudotaeniolina globosa* isolated from Djoser pyramid, Memphis necropolis, Egypt

Abstract

Most of rock-inhabiting fungi are meristematic and melanized microorganisms often associated to monument biodeterioration. In previous microbial profiling of the Egyptian Djoser pyramid, a *Pseudotaeniolina globosa* isolate was reported. The current study aimed to characterize at morphological, physiological, and molecular levels the *P. globosa* isolated from the Djoser pyramid compared with an Italian isolate. Experiments were carried out to test temperature, salinity, and pH preferences, as well as stress tolerance to UV radiation and high temperature, in addition to a multi-locus genotyping using ITS, nrSSU or 18S, nrLSU or 28S, BT2, and RPB2 markers. Morphological and molecular data confirmed the conspecificity of the two isolates. However, the Egyptian isolate possess a wider range of growth at different temperature being much more tolerant to heat (4–37 °C) and pH grades (3.0-9.0 pH) than the Italian one (10–30 °C, 4.0-6.0 pH), and more tolerant also to extreme salinity levels (5 M NaCl). The species was attributed to the family Teratosphaeriaceae, order Capnodiales. Our results demonstrated that the Egyptian isolate can be considered as a biovariant well adapted to harsh and extreme environment.

2.1 Introduction

Black meristematic fungi or microcolonial fungi (Staley, Palmar, & Adams, 1982, Wollenzien et al., 1995) that were more recently referred as “Rock-inhabiting fungi (RIF) (Rubial et al., 2009), are slow-growing melanized microorganisms often associated with natural rocky substrates (Sterflinger, 2000; Gorbushina, 2007). To economize the energy requirements in extreme environments, their life cycles are simplified (Selbmann, de Hoog, Mazzaglia, Friedmann & Onofri, 2005), lacking morphologically differentiated sexual phases, producing only a few metabolites and morphological structures crucial for survival (Sterflinger, 2006). This group of fungi possess peculiar characters related to stress tolerance, such as melanized cell walls, enabling them to successfully reside under harsh climatic conditions of prolonged desiccation, extreme temperatures (even in arctic habitats), high solar irradiation and osmotic stress, and limited nutrient availability (Selbmann et al., 2005, 2008), where cosmopolitan and fast-growing micro-fungi are unable to survive (Gorbushina, 2007). Even if the role of RIF in monument decay remained underestimated for a long time, it is now clear that they are among the most active groups of microorganisms causing weathering of rocks and biodeterioration of monuments exposed to outdoor conditions (Sert, Sümbül & Sterflinger, 2007; Sert & Sterflinger, 2010; Marvasi et al., 2012; Isola et al., 2013, 2016; De Leo & Urzì, 2015; Salvadori & Municchia, 2016); in addition to their consistent detection on marble monuments of the Mediterranean basin (e.g., De Leo, Urzì & de Hoog, 2003; Zucconi et al., 2012; Onofri, Zucconi, Isola & Selbmann, 2014; De Leo, Antonelli, Pietrini, Ricci & Urzì, 2019; De Leo, Antonelli, Pietrini, Ricci, & Urzì, 2019).

The taxonomic diversity of RIF appears to be unexpectedly wide, although only a limited number of RIF species and genera have been described (Egidi et al., 2014). Several studies have revealed that the ability of microfungi to grow on rocky substrates is a polyphyletic trait, assessing RIF in two different classes of Ascomycota, namely Eurotiomycetes (Order Chaetothyriales) and Dothideomycetes (mainly the Orders Capnodiales, Dothideales, and Pleosporales) (Sterflinger, de Hoog & Haase, 1999; Ruibal Villaseñor, 2004; Ruibal, Platas, & Bills, 2005, 2008; Gueidan et al., 2009;

Ruibal et al., 2009). Their identification also remained relatively intuitive because of their scarce differentiation and morphological plasticity, until molecular techniques became a common approach in fungal systematics; with the improvement of isolation procedures and molecular methods, it has become apparent that RIFs are actually much more common and widespread than previously believed (Ruibal et al., 2009).

Within the RIF group, *Pseudotaeniolina* J.L. Crane & Schokn. (Order Capnodiales) was introduced as a monotypic genus by Crane & Schoknecht (1986) for a species isolated from plant material named *P. convolvuli* (Esfand.) J.L. Crane & Schokn. De Leo et al. (2003) isolated the second species, *P. globosa* De Leo, Urzì & De Hoog, from the outside wall of the church of ‘‘Santa of Maria di Mili’’ in Messina, Italy. These authors described it as an anamorphic, melanized fungus with meristematic development followed by arthric secession leading preponderantly to the formation of single cells. The identification was supported by SSU (small subunit) and ITS (internal transcribed spacer) rDNA sequence data. However, the family name was still not assigned (Roskov et al., 2020). Currently, *P. globosa* has few isolates worldwide and a very limited dataset available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

Physical, chemical, and biological factors play a combined role in weathering archeological sites suffering from biodeterioration. Previously, in Egyptian monuments, species from genera *Aspergillus*, *Alternaria*, *Bipolaris*, *Cladosporium*, *Dichotomopilus*, *Fusarium*, *Rhizopus* and *Penicillium* were reported from several Egyptian archological remains (e.g., Archeological tombs in the Alexandria district (Afifi & Geweely, 2011); Tuna el-Gabel's excavations near Al-Minya city (Mansour & Ahmed, 2012); black spots from Great pyramid complex (Giza city), Mosque of judge Abd El basset (Cairo city), Museum of Ismailia Antiquities (Ismailia city), National Museum of Egyptian Civilization (Cairo city), Senusret I obelisk (Fayoum city), and Seti I tomb (Abydos city; Mohamed & Ibrahim, 2018)). Besides, a microbial survey performed in the Djoser pyramid complex using metabarcoding and traditional isolation methods reported *Pseudotaeniolina globosa* for the first time on

Egyptian archeological remains (see Chapter I), being the second worldwide report of the species after its description in Messina by De Leo et al. (2003).

This study aimed to deepen the last finding and had the following specific objectives: 1) to evaluate if the sample of *P. globosa* isolated from the step pyramid of Djoser correspond to the same isolate found previously in Messina or perhaps an extremotolerance biovariant; 2) in an affirmative case, to describe this biovariant, characterizing it at morphological, physiological and molecular levels and knowing its UV and temperature tolerance; 3) to better clarify the taxonomic position within the order Capnodiales based on multi-locus genotyping identification.

2.2 Materials and Methods

2.2.1 Isolate sampling

Pyramid of Djoser, also known as the “Step Pyramid”, is an archeological remain in the Saqqara necropolis, located in the northern part of the Nile Valley, situated at 29°52'10.17" N and 31°13'8.70" E in the Giza governorate, Egypt.

Two *Pseudotaeniolina globosa* isolates with the same morphological characteristics were isolated from soil particles collected at the Mastaba “Mehu” entrance and the Djoser pyramid's ground entrance using Dichloran Rose Bengal Chloramphenicol Agar (DRBC Agar) medium. The two isolates were found identical based on ITS sequences (see Chapter I), thus, one isolate only was used in the current study (coded as DPS10). The nomenclature of fungi follows Index Fungorum (http://www.indexfungorum.org).

2.2.2 Morphological characterization of *Pseudotaeniolina globosa*

Hyphal maturation and conidiogenesis, as well as culture media preference experiments, were performed with DPS10 isolate.

a) Hyphal maturation and conidiogenesis

They were studied using light and phase-contrast microscopy on slide cultures performed by inoculating squared agar blocks of Malt Extract Agar (MEA; #1038, Condalab, Spain). They were incubated for one month at 25 °C in wet sterile Petri dishes with filter paper to avoid media dehydrating, observed using lactophenol by Leica DMLB Tilting Trinocular Phase Contrast and Dark Field

Light Microscope. Digital images were captured using a Leica DFC500 digital color camera optimized with the software Micromax Arkon (v. 8.12.05).

b) Culture media preferences

Characteristics and growth rates of the colonies were studied and recorded after one month, performed in duplicates of divided Petri dishes of four sections, which contained MEA, Potato Dextrose Agar (PDA; #1022, Condalab, Spain), Oatmeal Agar (OA; #2060, Condalab, Spain), and Czapek Dox Agar (CzA; #1015, Condalab, Spain) and were incubated at 25 °C.

2.2.3 Physiological characterization of *Pseudotaeniolina globosa*

Temperature preferences, growth at different salt concentrations, and growth at different pH ranges, and tolerance experiments were all performed according to Selbmann et al. (2008). Colonies with diameter >2 mm were considered positive, according to Kane & Summerbell (1987).

In addition to the DPS10 isolate, isolate MC769 of *P. globosa* (CBS 109889^{T}) was used for physiological and molecular analyses. The last one was obtained from the fungal collection of the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Italy, and freshly re-cultivated using the culture medium Malt Extract Broth (MEB; #CM0057, Oxoid, USA).

a) Temperature preferences

Temperature preferences were tested using PDA Petri dishes divided into four sessions in duplicates, incubated at 4, 10, 25, 30, 34, and 37°C. The colonies diameter was recorded after one month of incubation to detect the optimum growth of the isolated isolate among the different selected temperature.

b) Growth at different salt concentrations

To test the ability of the fungus to grow in presence of different concentration of salts and to recognize its pattern of halotolerance, an experiment was performed by inoculating the DPS10 isolate on four spots in duplicates of MEA plates supplemented with a scale of NaCl concentrations of 1.2, 1.5, 3, 5, 7, 10, 12, 15, 18, 25, and 30%, incubated at 25 °C for one month.

c) Growth at different pH

The ability of growing at different pH values were tested in triplicates of MEB medium at pH levels of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. At standard conditions for RIFs pH growth (Selbmann et al., 2008), the experiment was initially performed at pH 5.0 by 1N HCl; then media was adjusted for pH 1.0 using HCl/KCl buffer and adjusted for pH 2.0–7.0 by using McIlvaine solution and adjusted for pH 8.0–9.0 by applying Clark & Lubs solution. DPS10 was incubated at 25 °C for one month in a shaken culture at 70 rpm (Küster & Thiel, 1999).

2.2.4 Tolerance assessment of *Pseudotaeniolina globosa*

As *P. globosa* was exposed to the sun and dryness prevailing in the Djoser pyramid area, it is supposed to be adapted to this arid environment. Therefore, an experiment was designed to recognize the extreme stress tolerance levels that the DPS10 and MC769 isolates can tolerate.

a) UV tolerance under wet and dry conditions

The ability of the fungus to survive and reproduce after the exposure to germicide UVC radiation (253.7 nm) in wet and dry conditions was tested. The experiment material was prepared in replicates by placing about 1 cm^2 mycelia of 30 days old culture in an open sterile Petri dish along with 1 ml physiological solution (0.9% NaCl), where the solution is not more than 1 mm depth (wet condition); or without the physiological solution (dry condition). Then the perti dishes were placed at 35 cm from the UVC lamp (Philips TUV T8 30W). The sampling was done in duplicates after 10 and 30 min, 1, 3, 6, 9, 12, and 24 h. The treated mycelia wet, and dry conditions were re-planted in PDA medium and incubated at 25 °C for one month to record their ability to regrow.

b) High-temperature tolerance

The experimental aim was to identify the degree of the fungus ability to survive and resume growth after the exposure of the cells to high-temperature stress. The experiment was performed in duplicates by harvesting about 1 cm^2 mycelia of 30 days old cultures of DPS10 and MC769 and transfer them to 1 ml of physiological solution in 2 ml tubes. The tubes were put in a water bath with adjusted to 65 and increased by 5 °C intervals til 85 °C, the sampling was done in duplicate for 2, 5, 10, and 15 min of exposure at each temperature. The same

was repeated in dry conditions using hot blocks without physiological solution. The treated mycelia from both wet and dry conditions were re-planted in PDA medium and incubated at 25 °C for one month.

2.2.5 Molecular characterization of *Pseudotaeniolina globosa*

Molecular characterization, comparison between the two isolates, and family assignment of *P. globosa* were performed using a PCR based approach combined with Sanger sequencing, commonly known as “multi-locus genotyping” using five molecular markers.

a) DNA extraction

DNA for both fungus isolates was extracted using cetyltrimethylammonium bromide (CTAB) acid washed beads using the manual protocol of Möller, Bahnweg, Sandermann, & Geiger (1992) and modified by Urzì, De Leo, Passo, and Criseo (1999). DNA was quantified with Qubit™ (Invitrogen, Life Technologies) and the Qubit™ dsDNA BR assay kit (Invitrogen, Life Technologies).

b) Multi-locus genotyping

The loci that were amplified for multi-locus comparison between the two isolates were the ITS, the small subunit of the nuclear ribosomal RNA (nrSSU or 18S), the large subunit of the nuclear ribosomal RNA (nrLSU or 28S), B-tubulin (BT2), and RNA polymerase II (RPB2; Egidi et al., 2014). Primers are listed in Table (2.1).

PCR reactions were performed using the MyTaq™ Red Mix (Cat# BIO-25043, BioLine, UK). Each 25 µl reaction tube included 5 pmol of each primer, and 40 ng of template DNA was added. The amplification was carried out using a Techne™ 512 thermocycler (Techne, UK). The PCR programs were adjusted according to the primer pair melting temperature (T_m) as follows: the first denaturation step at 95 °C for 5 min was followed by 33 cycles of denaturation at 95 °C for 30 s, annealing at the assigned temperature (see Table 2.1) for 30 s, extension at 72 °C for 30 s and a final extension 72 °C for 5 min.

Table 2.1 Multi-locus primers used for the identification of *Pseudotaeniolina globosa* from literature references.

Locus	Primer (Direction)	Annealing (°C)	Reference
ITS	ITS1 (F)	50	White, Bruns, Lee, & Taylor (1990)
	ITS4 (R)		
nrSSU	*NS1 (F)*	55	White et al. (1990)
	NS3 (F)		
	NS5 (F)		
	NS7 (F)		
	NS24 (R)		
nrLSU	LSU1Fd (F)	52	Crous et al. (2009)
	LR5 (R)		Vilgalys & Hester (1990)
BT2	T1	52	O'Donnell & Cigelnik (1997)
	T22		
RPB2	fRPB2-5F	49	Liu, Whelen & Hall (1999)
	fRPB2-5F+414R	49	Quaedvlieg et al. (2011)

PCR products were visualized using 1.5% agarose gel electrophoresis in 1x TBE buffer. All PCR reactions, when successful, were prepared for the cleanup step (purification) using GeneJET™ PCR purification kit (Fermentas, K0702) prior to automated Sanger sequencing. Chromatographs were trimmed, assembled, and aligned using Geneious Prime (Kearse et al., 2012) and blasted for species identification using NCBI online Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Retrieved sequences were revised and checked using GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore). Sequence were deposited in GenBank repository under the accessions number detailed in Table (2.2).

Taxonomic ranking and phylogenetic relationships were retrieved from the NCBI taxonomy database (https://www.ncbi.nlm.nih.gov/taxonomy). Alignments of the target sequences along with the BLAST query results were performed using ClustelW (Thompson, Higgins, & Gibson, 1994) using the default settings, while repeated sequences were discarded. Phylogenetic trees were constructed by the maximum likelihood method based on the Generalized Time-reversible (GTR) model using Fasttree v2.1.5 (Price, Dehal, & Arkin, 2010).

2.3 Results

2.3.1 Morphological characterization of *Pseudotaeniolina globosa*

a) Hyphal maturation and conidiogenesis

The fungal isolate was anamorphic. Colonies were growing slowly as they reach 21.0 mm in diameter after 30 days; yeast-like cell forms were absent, and the teleomorph was unknown. Black colonies were butyreae; with age, they became shiny of more rigid structure and wrinkled with cauliflower shape. The mycelium was composed of pale brown, thick-walled immersed branched hyphal cells, 7.0-12.0 µm long, and 4.0-6.0 µm wide. The meristematic mycelium eventually converted into multicellular clumps, 5.0-12.0 µm in diameter. Conidia were produced by arthric disarticulation of hyphae; they were uni- or bi-cellular, pale brown, constricted at the septa, 4.8-5.5 µm in diameter (Fig. 2.1).

b) Culture media preferences

The isolate DPS10 was able to grow and reproduce on different culture media after one month of incubation at 25 °C. On PDA, it showed a visible optimum growth with a diameter record of 21 mm, with black, shiny, hard, wrinkled structure and a cauliflower shape, slightly raised at the center (umbonate), radially folded, with a unique appearance of an olive-green glow surrounding the colony edge. On MEA and CzA media, it showed a small reduction of 19.5 mm in diameter, while the same morphological appearance was observed, except an olive-green color on MEA and change from umbonate to crateriform with leathery texture on CzA. The smallest ultimate colony diameters were observed on the OMA medium, having 18.5 mm, with a different appearance of flat, hairy, gray growth on the colony top. No changes in the conidia morph among the tested media were observed (Fig. 2.2). As reported by De Leo et al., (2003) MC769 isolate colony on PDA showed the highest growth record of 28.0 mm in diameter with flat, regular margin, slightly raised at the center, cerebriform, radially folded. On MEA, the colony was black and shiny, buttery, flat slightly raised at the center of 25.0 mm in diameter. On OMA, it had a sharp, regular margin of 21.0 mm in diameter, while the colony on CzA was flat, with a fimbriate margin, attaining up to 10.0 mm diameter (Fig. 2.2).

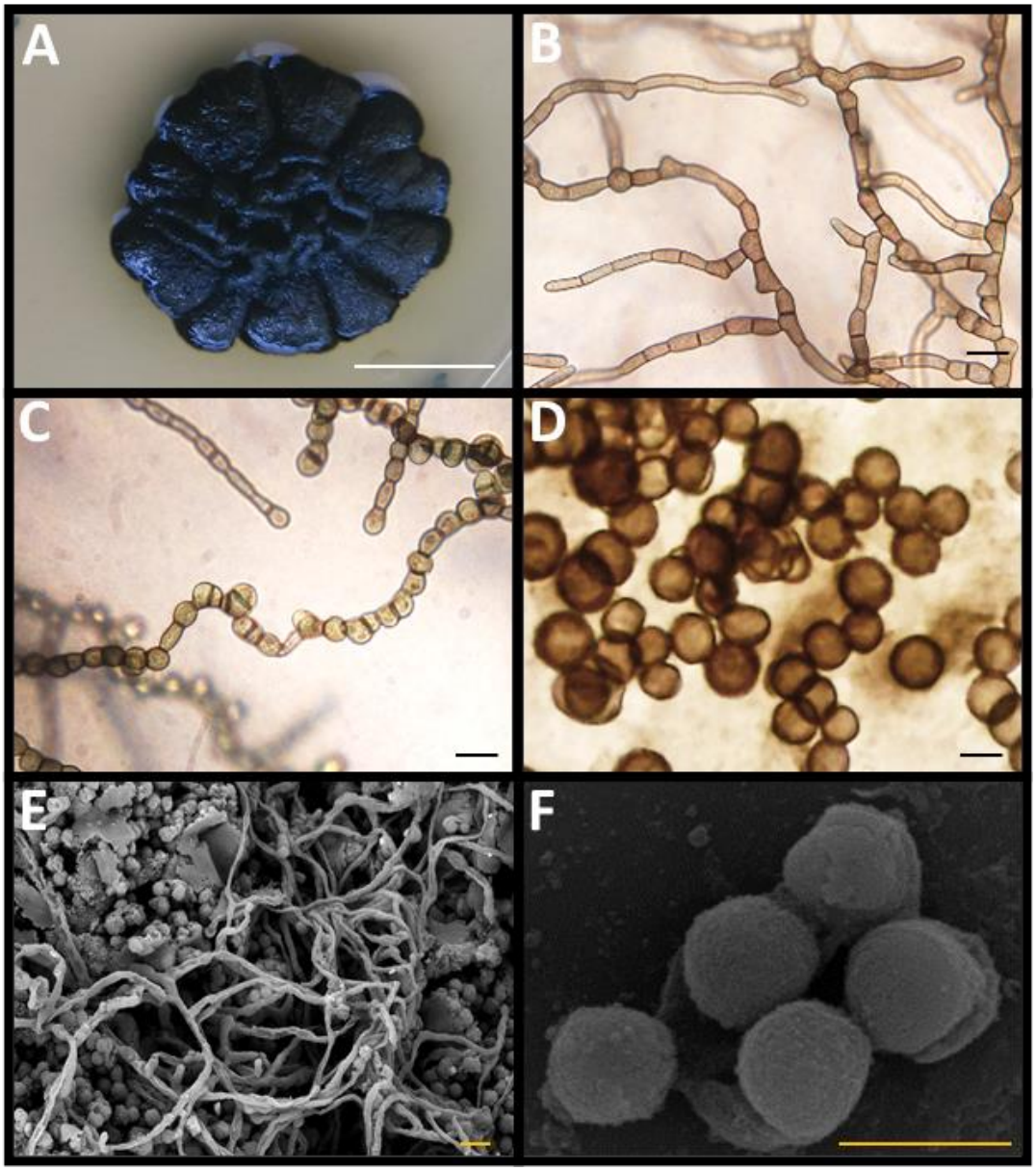


Fig. 2.1 *Pseudotaeniolina globosa* DPS10 isolate morphology. A: colony after one-month incubation on MEA medium. B: meristematic hyphal mycelia. C: clumps of meristematic structure. D: mature spherical conidia. E: hyphal structure and mature conidia. F: mature conidia (A to D: micrographs under the light microscope; E-F: micrographs under a Scanning electron microscope. White bar = 1 cm, black bars = 20 µm, orange bars = 10 µm).

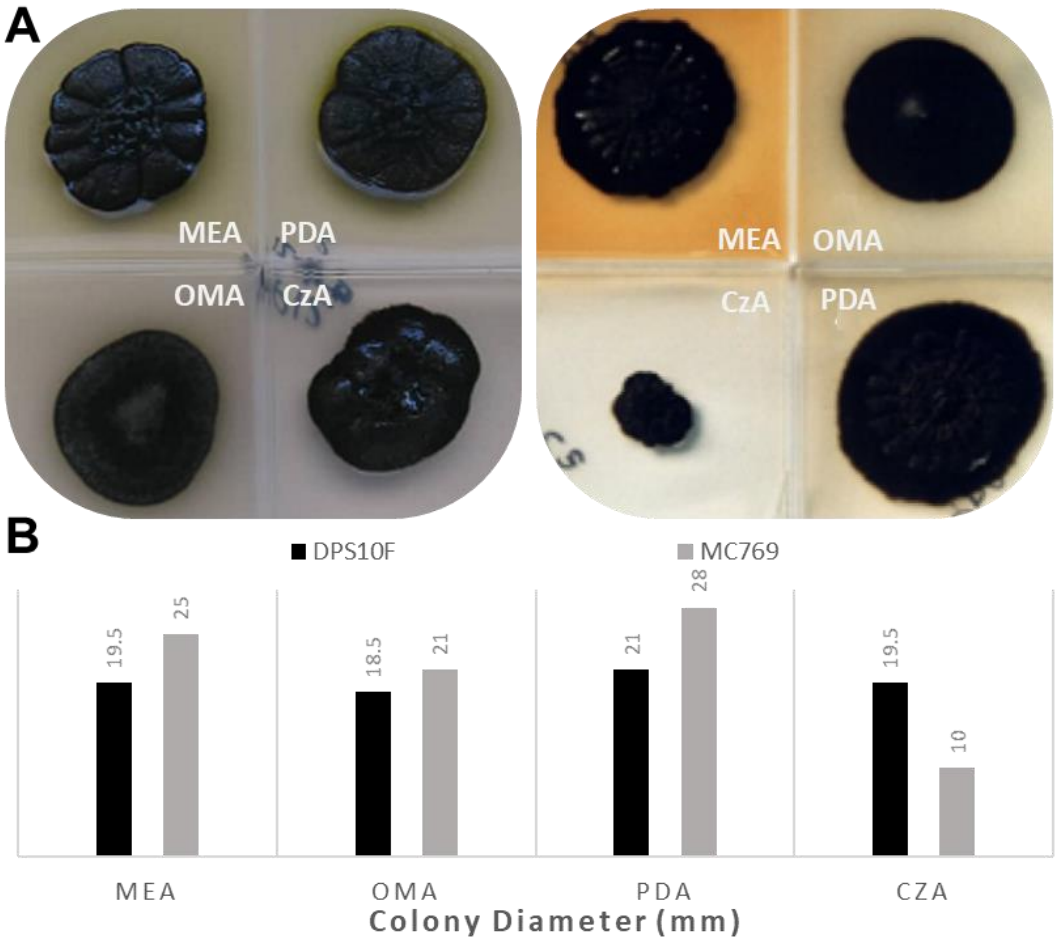


Fig. 2.2 Colonies of *Pseudotaeniolina globosa* grown on different media (MEA, OMA, PDA, CzA). A: colonies of DPS10 isolate (left) and MC769 isolate (right). B: colonies diameter (MC769 isolate data as describe by De Leo et al., 2003).

2.3.2 Physiological characterization of *Pseudotaeniolina globosa*

a) Temperature preferences

The DPS10 isolate was able to grow and reproduce at a wide range of temperatures, ranging from 4 to 37 °C, with the optimum growth was observed at 25°C where the colony reached 21.0 mm in diameter; above this temperature, a considerable reduction of colony diameter occurred at 30°C with 13.7 mm and at 37°C with 4.8 mm in diameter. The lowest temperature at which the colony was able to grow was at 4 °C, reaching 7.0 mm in diameter. Considering these data, it can be referred to as a mesophilic-psychrotolerant fungus. On the contrary, as reported by De Leo et al. (2003), the isolate MC769 showed the same diameter of optimum growth at 25°C and near the same diameter between 10 to 30 °C, without any record of growth at lower and higher temperature degrees (Fig. 2.3).

b) Growth at different salt concentrations

The DPS10 isolate was able to grow at all the tested NaCl concentrations, even at 30%, the maximum checked. It showed colonies of 22.0 to 18.5 mm diameter until 10% of NaCl concentration and showed a limited reduction of colony diameter of 17.0 and 14.6 at 12% and 15% NaCl concentration, respectively; and a considerable reduction to 9.5 and 7.5 at 18% and 30% NaCl concentration, respectively. Differently, MC769 isolate only grew at a maximum of 1.20% NaCl concentration according to the data adopted from De Leo et al. (2003; Fig. 2.4).

c) pH growth ranges

The DPS10 isolate showed an ability to grow at a wide range of pH values (from pH 3.0 to pH 9.0). The maximal growth was recorded between pH 5.0 and pH 7.0, and the minimal growth was recorded at pH 3.0 and pH 9.0. Based on the data from De Leo et al. (2003), MC769 showed growth at a narrower range of pH values (from pH 4.0 to pH 7.0), with maximal growth at pH 5.0 and minimal growth at pH 7.0. Comparing the two isolates, both started to grow at pH 4.0 with optimal growth up to pH 5.0 for MC769 and up to pH 7.0 for DPS10 and continued with growth reduction to pH 9.0. On the contrary, MC769 showed no growth at pH 8.0 and pH 9.0 (Fig. 2.5).

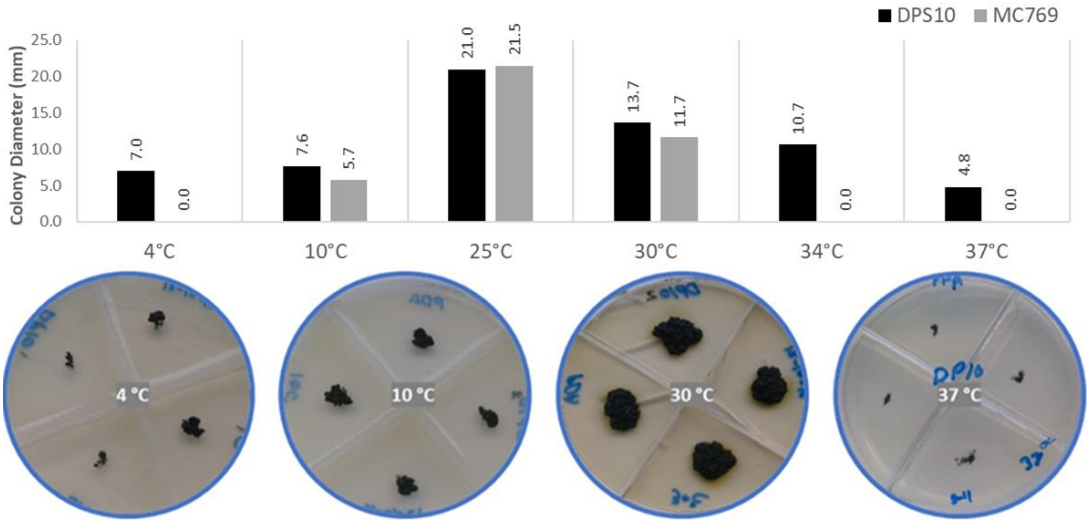


Fig. 2.3 Temperature preferences of *Pseudotaeniolina globosa* DPS10 isolate shown by the colony diameter reached on PDA medium after one-month incubation. The column chart in the upper part exhibit comparison of DPS10 and MC769 isolates at 4, 10, 25, 30, 34, and 37°C. Visual examination of the colonies in the lower part shows the growth of DPS10 at 4, 10, 30, and 37 °C in quadruplicates (Petri dish of four divisions). Data on MC769 isolate come from De Leo et al. (2003).

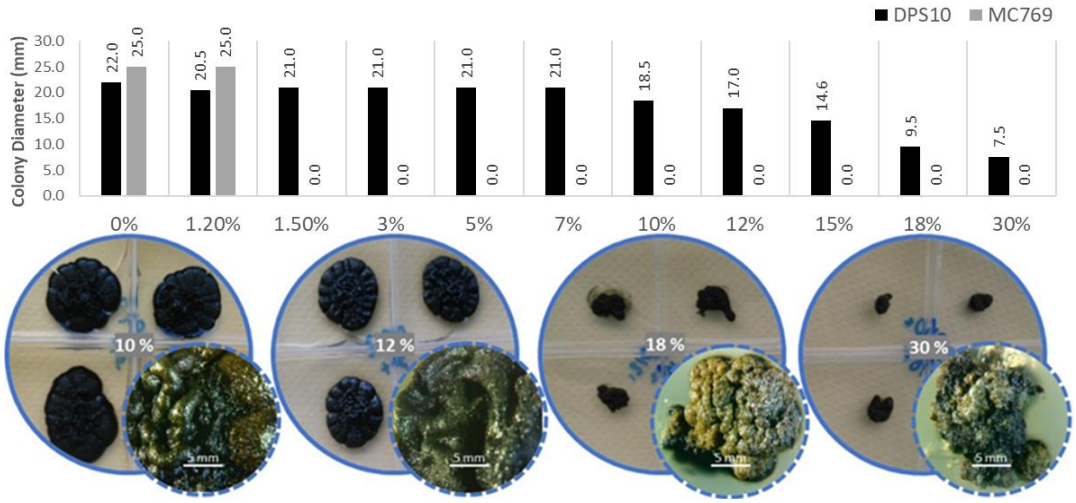


Fig. 2.4 Salinity preferences of *Pseudotaeniolina globosa* DPS10 isolate shown by the colony diameter reached on MEA medium after one-month incubation at 25 °C. Column chart in the upper part exhibit comparison of DPS10 and MC769 isolates in medium supplemented with 0, 1.2, 1.5, 3, 5, 7, 10, 12, 15, 18 and 30% NaCl. Visual examination of the cultured plates in the lower part shows the growth of DPS10 at 10, 12, 18, and 30% NaCl in quadruplicates (Perti dish of four divisions) with a stereomicroscopic focus for one of the grown colonies (bar = 5 mm). Data on MC769 isolate as reported by De Leo et al. (2003).

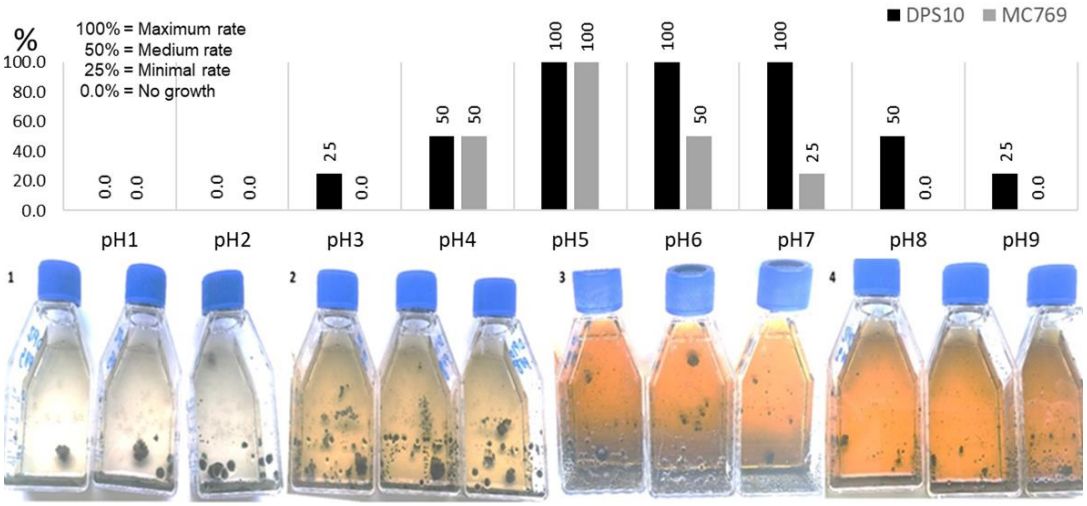


Fig. 2.5 The pH growth range of *Pseudotaeniolina globosa* DPS10 isolate shown by four established growth grades (0, 0.25, 0.5, 1) reached on MEB medium after one-month incubation at 25 °C. Column chart in the upper part exhibit comparison of DPS10 and MC769 isolates measured at pH 1.0 to pH 9.0. Flasks in the lower part show growth at pH 4.0 (1), pH 6.0 (2), pH 8.0 (3), and pH 9.0 (4). Data on MC769 isolate are those reported by De Leo et al. (2003).

2.3.3 Tolerance experiments of *Pseudotaeniolina globosa*

a) UV tolerance under wet and dry conditions

The isolates DPS10 and MC769 showed an equal maximum growth rate after UV exposure of 10 min until 6 h; the isolates maintained the same colony diameter (21.0 and 25.0 mm, respectively). MC769 showed a reduction of 50% of average colony diameter (after 9, 12, and 24 h of UV exposure), while DPS10 showed the 50% reduction after 12 and 24 h of UV exposure (Fig. 2.6).

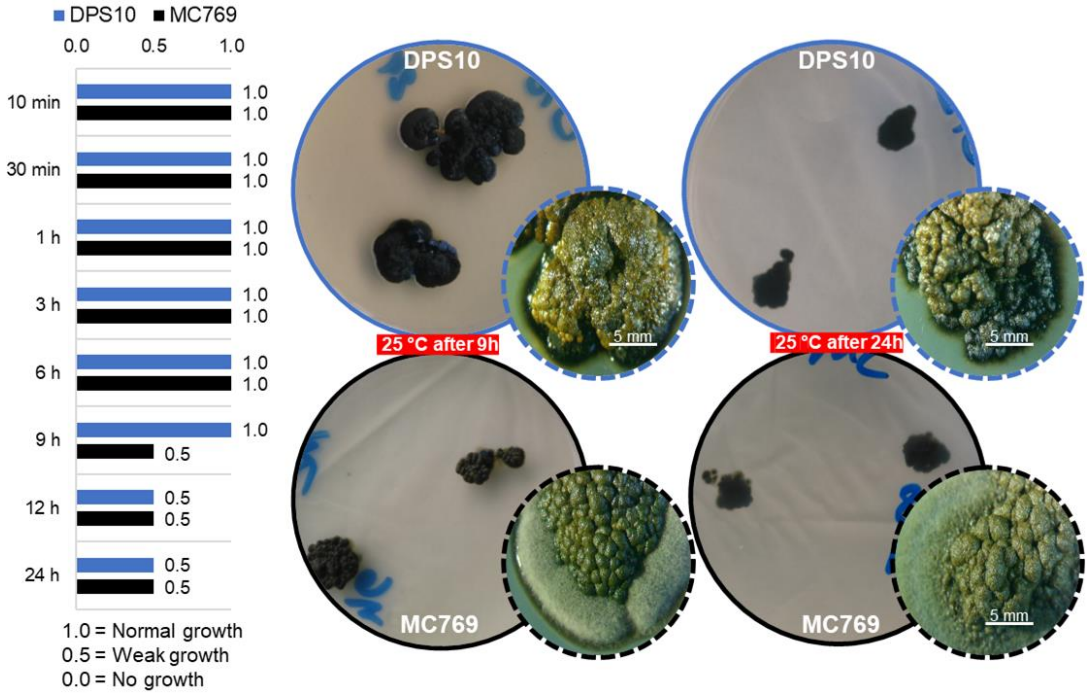


Fig. 2.6 Tolerance examination of *Pseudotaeniolina globosa* DPS10 and MC769 isolates after exposure to UV. Column chart on the left side part exhibit comparison of DPS10 and MC769 isolates growth monitored on PDA media at 25 °C after 10 and 30 min, 1, 3, 6, 9, 12, and 24 h of exposure. Visual examination of the cultured plates in the right part shows the growth of DPS10 and MC769 isolates after 9 and 24 h, with a stereomicroscopic focus for one of the grown colonies (bar = 5 mm).

b) High-temperature tolerance

Heat stress on isolates DPS10 and MC769 at 65, 70, and 75 °C after 2 and 5 min showed the maximum growth rate a colony can reach in 1 month (colony diameter of 21.0 and 25.0 mm, respectively). However, when the exposure extended to 10 and 15 min a lower growth rate was observed to all the tested temperatures for both isolates. At 70 and 75 °C, after 15 min of exposure, the MC769 showed a slow rate than the low rate described for DPS10. When the

80 °C was tested, both isolates were grown at low rate after 2 and 5 min of exposure, however, only DPS10 maintained the same rate after 10 min and showed a slow rate after 15 min of exposure in contrast to MC769 which showed no growth. When the 85 C was tested, only DPS10 was grown at a slow rate after 2 and 5 min, while showed no growth after 10 and 15 min of exposure (Fig. 2.7).

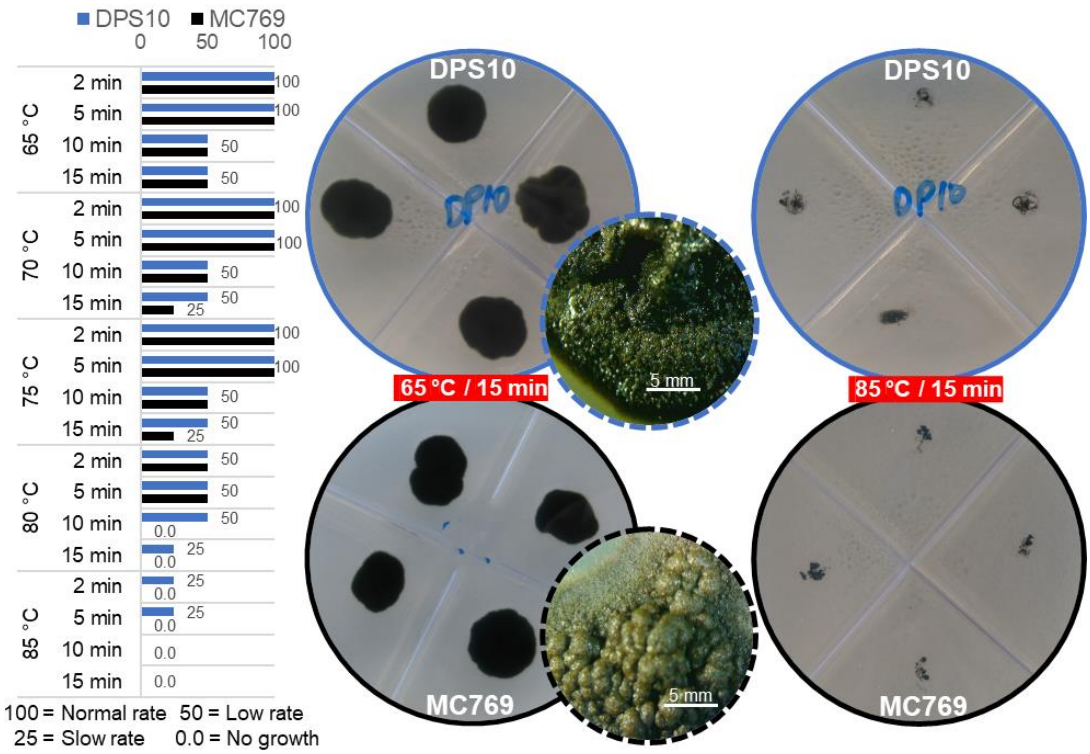


Fig. 2.7 Tolerance examination of *Pseudotaeniolina globosa* DPS10 and MC769 isolates after heat exposure. Column chart in the left part exhibit comparison of DPS10 and MC769 isolates growth monitored on PDA media after 2, 5, 10, and 15 min at 65, 70, 75, 80, and 85 °C; the x-axis represents the grade from 100 for normal growth rate after 1 month of incubation (21.0 and 25.0 mm colony diameter for DSP10 and MC769, respectively) to 0.0 for no growth. Visual examination of the cultured plates in the right part shows the growth of DPS10 and MC769 at 65, and 85 °C after 15 min of heat exposure, with a stereomicroscopic focus for one of the grown colonies (bar = 5 mm).

2.3.4 Molecular characterization and phylogeny of *Pseudotaeniolina globosa*

The amplification of five loci for the two isolates was successful, showing the expected molecular size as reported previously. Based on the BLAST results, similar top accessions were retrieved and filtered by query coverage (i.e., < 95% query coverage were discarded) and matched with “Popsets” files downloaded from the NCBI database for each locus prior to alignment and phylogenetic analysis. All the retained accessions belonged to the order Capnodiales. The ML phylogenetic trees were rooted with the Extremaceae family species, while all the remaining sequences belonged to the family Teratosphaeriaceae and Mycosphaerellaceae for all the genotyped loci.

In the case of ITS, a total of 30 accessions belonging to the order Capnodiales in addition to the current two isolates of *P. globosa* (DPS10 and MC769) showed an alignment length of 566 bp, including partial 5’ and 3’ termini of both ITS1 and ITS2, respectively, and the complete 5.8S locus. The number of identical sites was 253 bp (50%), while the un-gapped average length was 446 bp with GC% = 55.9%. A major observation is that the family members Teratosphaeriaceae were highly clustered into two sub-clades (I and II; bootstrap value ≥ 0.80). The isolate *P. globosa* DPS10 was highly clustered to eight *P. globosa* accessions (bootstrap value = 0.96). The most similar sequence to the Egyptian isolate (with bootstrap value = 0.81) was the specimen CBS30384, which was early registered in GenBank as *Trimmatostroma* sp. (de Hoog et al., 1999) and corrected to *P. globosa* in later publications; Selbman et al., 2008). The geolocation of the samples (Table 2.2) does not reflect the phylogenetic relationships based on this marker (Fig. 2.8, ITS).

In the case of the nrSSU (18S), a total of 18 accessions belonging to the order Capnodiales in addition to the current two isolates of *P. globosa* (DPS10 and MC769) showed an alignment length of 1,670 bp. The number of identical sites was 1,541 bp (92.3%), while the un-gapped average length was 1667 bp with GC% = 48.5%. The *P. globosa* isolates were included within the clades identified as order Capnodiales. In addition to DPS10 and MC769 isolates, a single *P. globosa* accession CCFEE 5734 was highly clustered in the same

clade (bootstrap value = 0.92). The *P. globosa* accessions were sister to *Hortaea werneckii* (Horta) Nishim. & Miyaji (CBS 107.67), a very peculiar species known as an extremophilic halotolerant fungus, and *Stenella araguata* Syd. (CBS 105.75) (family Mycosphaerellaceae) a biological causal agent for Tinea nigra dermatological disease (bootstrap value = 0.79; Fig. 2.8, nrSSU 18S; Perez et al., 2005).

For the nrLSU (28S), a total of 28 accessions in addition to the current two isolates of *P. globosa* (DPS10 and MC769) showed an alignment length of 782 bp (incomplete sequence, partial at 3' terminal). The number of identical sites was 573 bp (73.8%), while the un-gapped average length was 760 bp with GC% = 53.9%. The ML tree showed a monophyletic clustering of the families within order Capnodiales with the Extremaceae clade as root, followed by the Mycosphaerellaceae clade and the Teratosphaeriaceae clade. In the latter, the *P. globosa* DPS10 and MC769 isolates were highly clustered with two *P. globosa* accessions recorded as Capnodiales *incertae sedis* (bootstrap value = 0.99; Fig. 2.8, nrLSU 28S).

Based on BT2, all retrieved sequences by BLAST without the intronic regions were discarded. A total of nine accessions and the current two isolates of *P. globosa* (DPS10 and MC769) showed an alignment length of 425 bp. The number of identical sites was 197 bp (48.2%), while the un-gapped average length was 385 bp with GC% = 55.6%. The isolates of *P. globosa* were included within the clades identified as order Capnodiales, family Teratosphaeriaceae (Fig. 2.8, BT2).

In the case of RPB2, a total of 19 accessions and the current two isolates of *P. globosa* (DPS10 and MC769) showed an alignment length of 239 bp. The number of identical sites was 107 bp (44.8%), while the un-gapped average length was 239 bp with GC% = 53.1%. The isolates of *P. globosa* were included within the clades identified as order Capnodiales, family Teratosphaeriaceae with bootstrap value = 0.70 (Fig. 2.8, RPB2).

Table 2.2 List of *Pseudotaeniolina globosa* available accessions in the GenBank database with an indication of the literature reference where they were published.

Isolate code	Isolation country	GenBank accession number					References
		ITS	nucSSU (18S)	nucLSU (28S)	BT2	RPB2	
CBS 303.84*	Germany	AJ244268	-	-	-	-	de Hoog et al. (1999)
CBS 110353	Germany	AJ244268**	-	-	-	-	Kurzai, Keith, de Hoog, Abele-Horn & Frosch (2003)
MC769 CBS 109889^{T}	Italy***	AY128700	NG062782	-	-	-	De Leo et al. (2003)
L10	Austria	HQ115663	-	-	-	-	Gorfer et al. (2011)
ICP 1002	Austria	KC311489	-	-	-	-	Gorfer, Klaubauf, Berger & Strauss (2014)
CCFEE 5734	Italy	KF309976	-	KF310010	KF546758	KF310073	Egidi et al. (2014)
H19	Chili	KF578436	-	-	-		Ortiz et al. (2014)
HF24	Austria	KR081416	-	-	-	-	Piñar, Dalnodar, Voitl, Reschreiter & Sterflinger (2016)
DPS10	Egypt	MH396690	MH396869	MH396691	-	-	Current study

* Initially identified as *Trimmatostroma* sp.
** No sequences were reported but mentioned in the reference to be similar to CBS 303.84.
***First report and description of the species.

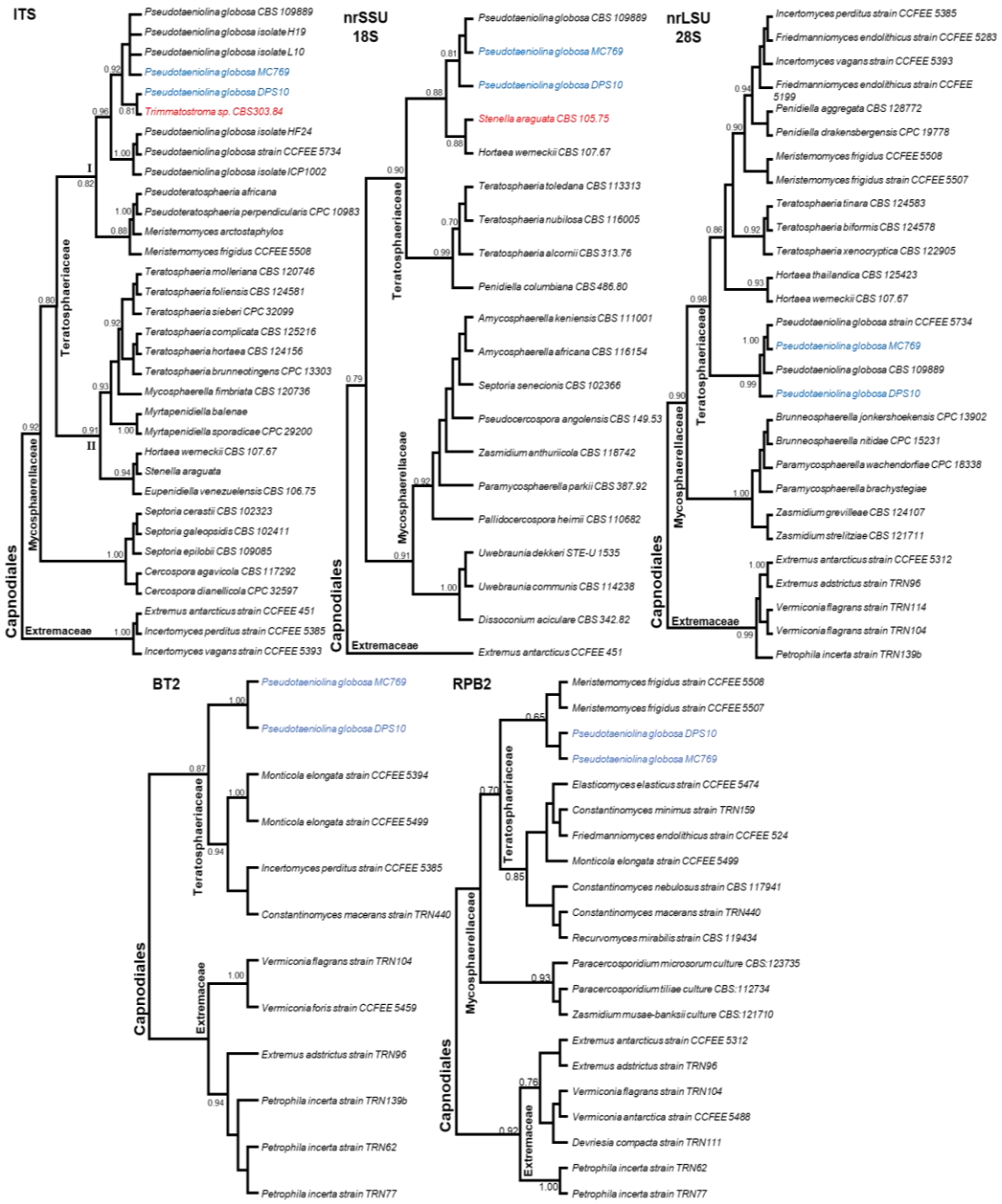


Fig. 2.8 Maximum likelihood-based phylogenetic trees for top similar accessions of Extremaceae, Mycosphaerellaceae, and Teratosphaeriaceae families (order Capnodiales) from GenBank NCBI database to ITS, nrSSU 18S, nrLSU 28S, BT2 and RPB2 sequences of *Pseudotaeniolina globosa* isolates (MC769 and DPS10 written in blue color). Trees are rooted by the Extremaceae clade/species; mistakenly labeled/grouped accessions are written in red.

2.4 Discussion

Black meristematic fungi or RIFs are remarkably extremotolerant microorganisms, frequently isolated from stone surfaces and habitats where minimal nutrients are available (Urzì, De Leo, de Hoog & Sterflinger, 2000). RIF not only survive under extreme conditions, but also grow to form microcolonial fungal life forms (MCF; Gorbushina, 2003). The selective pressure of such harsh conditions directed the evolution of their genomes to develop tolerant alleles through adaptive genetic variation and accumulation of beneficial mutations; the genetic pools of those organisms deviated significantly from sister fungal lineages to overcome the rock surface conditions (e.g., high UV radiation and temperatures; Ametrano, Muggia & Grube, 2019). For instance, the highly concentrated black pigments in the MCF cell walls serves as a UV-protective substance (e.g., Urzì, Wollenzien, Criseo, & Krumbein, 1995).

Based on our previous work, a RIF isolate identified as *Pseudotaeniolina globosa* using ITS sequences and coded DPS10 was recorded from the Djoser pyramid complex area and had never been reported on Egyptian archaeological remains. *Pseudotaeniolina globosa* is one of the uncommonly recorded RIF; only a few accessions were reported previously. All the reported isolates are known as environmental saprobes, predominantly found in water-limited ecological niches. Except for a unique accession that was isolated from the aortic wall of a patient with an aortic aneurysm (Kurzai et al., 2003).

In the current work, the morphological measurements of the hyphal length and width on MEA media were smaller than the MC769, however, morphologically, the DPS10 produce single cells or asymmetrically septate cells after conidiogenesis which is a unique character to *P. globosa* species (De Leo, 2003). The current isolate is slimy and form a yeast-like colonies after 4-5 days of growth, which differentiate the *P. globosa* from a micro-morphologically similar species known as *Sarcinomyces petricola* Wollenzien and De Hoog (Wollenzien, de Hoog, Krumbein, & Uijthof, 1997; De Leo et al., 2003). Regardless of the morphological variation between both isolates, the molecular characterization of the DPS10 isolate confirms its identity and high similarity to the *P. globosa* MC769 isolate. However, under abiotic stress (pH, salinity, and temperature) the DPS10 showed tolerance to extreme conditions.

The pH of a medium is a determined factor for fungal presence and diversity, for example, the soil pH was the most significant factor correlated to the fungal community composition in Svalbard, High Arctic (Kaštovská, Elster, Stibal, & Šantrůčková, 2005). The DPS10 optimum growth was at 5.0-7.0 pH and grew on a broader range from 3.0 to 9.0 pH compared to MC769, which grew well between 4.0-6.0 and scarcely at 7.0 pH. Thus, the ability of DPS10 to colonize different media with different pH ranges worth further studying.

The DPS10 optimum growth was at 0.25 M (1.5%) and continued growth, although at lower rates, up to 5.2 M (30%) NaCl supplemented MEA, while MC769 was strictly limited to 0.20 M (1.2%). Fungi are considered halophilic if they are isolated from sites with 1.7 M (~10%) and halotolerant if its sporadic isolates can grow *in vitro* at 3 M NaCl supplemented medium (Gunde-Cimerman, Ramos, & Plemenitaš, 2009). According to previous reports of model halotolerant fungi, *Debaryomyces hansenii* (Zopf) Lodder & Kreger-van Rij in up to 3 M (Prista, Loureiro-Dias, Montiel, García, & Ramos, 2005), *Hortaea werneckii* in up to 5 M (Plemenitaš, Vaupotič, Lenassi, Kogej & Gunde-Cimerman, 2008), while *Wallemia ichthyophaga* Johan-Olsen thrives in 5.2 M, the latter of which is considered the most halophilic fungus, however it is unable to grow without salt in its medium (Zalar, de Hoog, Schroers, Frank & Gunde-Cimerman, 2005). The MC769 isolate was previously described as halo-sensitive (De Leo et al., 2003); however, according to our results, the DPS10 isolate can be considered as halophilic and halotolerant (Gunde-Cimerman et al., 2009). At the level of intracellular Na and K ion accumulation, it was shown that *D. hansenii* and *W. ichthyophaga* are Na^+-intruder fungi and accumulate higher amounts of Na ions than *H. werneckii*, which excludes these ions from their cells (Gunde-Cimerman et al., 2009). Halophily is expressed in several groups of the same order but not the closest at the taxonomic level (Gunde-Cimerman et al., 2009). *Hortaea werneckii* is the nearest phylogenetic neighbor to *P. globosa* and most probably follows the same mechanism. Alternatively, they are extreme xerotolerant whose growth on substrates is determined by water potential and not by the chemical nature of the solute (Pitt & Hocking, 2009).

Both biovariants, DPS10 and MC769 showed optimum growth rate at 25 ºC, but DPS10 had a broader range of growth temperatures (4–37°C) than MC769 (10–30 ºC). Additionally, DPS10 isolate was able to regrow after being exposed to 85 ºC for 5 min. Both *P.* globosa isolates are melanized, thus constitute chemical ability to absorb and tolerate UV light (Grishkan, 2011). Both isolates were tolerant to UV exposure up to 24 h; however, the growth rate was not affected up to 9 h in the DPS10 case compared to MC769 that was affected after 9 h of exposure to UV. Melanin not only serves as an extracellular electron-dense granular layer for black fungi and yeasts but also form an intracolonial matrix structure (Gorbushina, 2003). The results suggest that the DPS10 is an extremotolerant *P. globosa* biovariant adapted to the harsh and extreme environment as the arid and desert climate; can survive pH changes, high salt concentrations, tolerate high temperatures and UV radiation. It currently colonizes stones with cultural heritage value, in our case, the Djoser pyramid of Egypt.

Within Ascomycota, the main orders with halophilic and halotolerant representatives are Capnodiales, Dothideales, and Eurotiales (Gunde-Cimerman et al., 2009). Among them, the Capnodiales have a xerotolerant tendency, as they contain a large number of extremotolerant species that can grow as epilithic or cryptoendolithic species at high or low temperatures (Selbman et al., 2005). Most RIF possesses convergent morphological and physiological characteristics; however, many subgroups of RIF are phylogenetically uncertain within the class Dothideomycetes (Ruibal et al., 2009). According to the NCBI taxonomy database, the genus *Pseudotaeniolina* is uncertainly positioned within the order Capnodiales (*incertae sedis*) with no family assigned. When first reported, *P. globosa* was found clustered with species associated with low water availability (halophilic, epilithic or epiphytic) and defined as RIF within the Dothideales but was poorly supported by the phylogenetic analysis using ITS sequences (>50%; De Leo et al., 2003). In the current study, based on five molecular markers (ITS, SSU, LSU, BT2, and RPB2), *P. globosa* was clustered within the Teratosphaeriaceae family with bootstrap value > 0.7 – 0.99.

Ruibal et al. (2009) conducted a phylogenetic analysis using a multi-locus approach (nucSSU, nucLSU, and mtSSU) to resolve several taxonomic complexes of Dothideomycetes families. Among other families, the Teratosphaeriaceae was the most diverse and phylogenetically conflicting. The family Teratosphaeriaceae was represented as two separated clades, numbered as (1) and (2); however, the study did not include any *P. globosa* accession. When we matched the species, the *P. globosa* should be part of Teratosphaeriaceae group (1). The clustering was apparent at the ribosomal cistern sequences (ITS, SSU, and LSU) when conducted separately as resulted in a better resolution for the family compared to the closest one (Mycosphaerellaceae), especially the LSU. In contrast to other markers, the SSU phylogenetic signal was the only marker to show a paraphyletic clustering among the two proximate families, Mycosphaerellaceae and Teratosphaeriaceae; however, the taxonomical resolution within the Teratosphaeriaceae was not as structured as the ITS based phylogeny. The current study focused on the taxonomical family assignment of the *P. globosa* species rather than the taxonomical analysis of the order or finding the best marker for such an assignment. However, many accessions were discarded and missing in our analysis (e.g., RPB2) due to wrong labeling or incomplete metadata about the accessions, which require urgent revision to avoid misleading phylogenetic signals. For instance, the *P. globosa* CCFEE 5734 RPB2 sequence (GenBank accessions KF310073) that seems to be confused with *Extremus antarcticus* Quaedvl. & Crous isolate CCFEE 451 RPB2 sequence (GenBank accessions KF310085; Egidi et al., 2014).

Our prospective analysis will target the genomic signatures of the extremotolerance characters of the DPS10, to accurately detect the transcriptional responses and metabolic suspension and reactivation under the fluctuation of water availability and its extreme ability to maintain viability in a high salinity medium. Which will be achievable with more detailed comparison at genomic, transcriptomic, and metabolic levels using NGS-based approaches.

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CHAPTER III

*aro*F and *cm*2: new potential molecular markers for the detection of stone-inhabiting Actinobacteria on cultural heritage sites

Abstract

Tangible archaeological sites and stone monuments are naturally decayed and deteriorated over time, providing substances that can sustain life, although they provide a complicated ecosystem characterized by low nutrition and desiccation. Stone-inhabiting bacteria (SIB) and especially members of the phylum Actinobacteria dominate such environments, particularly the members of the family Geodermatophilaceae. We used the published data of two confirmed SIB species to mine their genomes for specific molecular markers to rapidly survey the presence of SIB in cultural heritage material prior to further analysis. The search focused on the mycosporine-like amino acids (MAAs) synthesis pathway. MAAs are intracellular compounds biosynthesized by the shikimic acid pathway to synthesize aromatic amino acids and were found related to abiotic resistance features in microorganisms. Based on genome mining, one candidate gene was a homolog to a key gene in the MAAs, and shikimate pathways and is known as DAHP II (*aro*F), and the other candidate gene was a homolog of the Chorismate mutase gene (*cm*2). Both were found mostly in Actinobacteria and few other species. After calibration on five stone-inhabiting Actinobacteria (SIAb) species using conventional PCR, the newly designed primers were successfully applied to the environmental DNA extracted from two Egyptian pyramidal sites using a qPCR approach. This is the first report of *aro*F and *cm2* as qPCR markers to detect SIAb from cultural heritage material prior to proceeding with further analysis (e.g., metagenomics and metabarcoding analyses).

3.1 Introduction

Archaeological sites and stone monuments represent a valuable and important part of the cultural heritage worldwide. A large percentage of the world's tangible cultural heritage is made from stone, and it is slowly but irreversibly disappearing by the transformation of the stone into sand and soil as a part of the natural recycling process, essential to sustain life on earth (Allsopp, Seal, & Gaylarde, 2004; Gadd, 2017). Despite the harsh conditions resulting from low water availability and nutrient concentration, stone surfaces represent a complex ecosystem consisting of several microhabitats, which enable a diverse range of microorganisms to proliferate. Besides algae, lichens, and fungi, cyanobacteria, actinobacteria, and other bacteria were reported to form the complex microflora of the stone surface (e.g., Urzì, De Leo, de Hoog, & Sterflinger, 2000; Scheerer, Ortega-Morales, & Gaylarde, 2009; Pena-Poza et al., 2018). For instance, *Micrococcus* Cohn 1872 sp. was isolated from deteriorated monuments and showed negative consequences on stone minerals due to some by-products from its metabolism (Tiano, Biagiotti, & Mastromei, 1999; Lo Schiavo, De Leo, & Urzì, 2020).

Stone-inhabiting bacteria (SIB) are peculiar bacterial species, especially members of the phylum Actinobacteria (SIAb) that mostly dominate this environment (Stackebrandt, Rainey, & Ward-Rainey, 1997). Within Actinobacteria, species of the family Geodermatophilaceae were previously reported as SIB (Urzì et al., 2001), causing a deteriorating aspect on stones in outdoor conditions (e.g., colored spots as well as biopitting and powdering; Urzì & Realini, 1998; Sghaier et al., 2016). Those species have a complex life cycle and produce remarkably resistant enzymes (e.g, esterases; Essoussi et al., 2010; Jaouani et al., 2012). They also can resist UV light, ionizing radiation, desiccation, and heavy metals (Rainey et al., 2005; Gtari et al., 2012; Montero-Calasanz et al., 2014, 2015). Their isolation *in vitro* is not easy and are often underestimated because the cultivation method needs at least 15 days of incubation and appropriate media (Urzì, Salamone, Schumann, Rohde, & Stackebrandt, 2004). Thus, a fast and reliable molecular method to detect SIAb on cultural heritage material would be an essential tool in the field of the applied cultural heritage microbiology.

Physiological and morphological approaches were extensively used to describe the endolithic microbial biodiversity and extended to the 16S rRNA sequencing-based techniques, such as species identification, PCR-denaturing gradient gel electrophoresis (PCR-DGGE), metabarcoding, metagenomics and quantitative PCR (qPCR), in which some specific bacterial strains have been consistently reported from different bio-deteriorated stone and monuments (e.g., Urzì et al., 2001). For example, fluorescence in situ hybridization technique was previously applied to distinguish members of Geodermatophilaceae family (Urzì, Lacono and Stackebrandt, 2004). *Geodermatophilus* Luedemann 1968, *Blastococcus* Ahrens & Moll 1970, and *Modestobacter* Mevs et al. 2000 emend. Qin et al. 2013 are three genera of the family Geodermatophilaceae and were described using 16S rRNA restriction analysis (Urzì et al., 2004). The 16S rRNA is extensively used to identify and quantify bacteria at familiar, generic, and specific levels using Sanger sequencing and qPCR techniques (e.g., del Mondo, de Natale, Pinto, Pollio, 2019). Advancements in PCR technology allowed for bacterial quantification using 16S rRNA qPCR. The qPCR allowed the microbial quantification in agriculture, environmental and medicine-related samples (Zhang & Fang, 2006). However, until recently it was frequently not successful for cultural heritage material (Liu et al., 2018). 16S rRNA qPCR specific primers were previously reported to detect Geodermatophilaceae members on the stone surface. However, the amplification did not include the other stone-inhabiting Actinobacteria (Salazar, Valverde, & Genilloud, 2006). Exploring more molecular markers is not frequent in the field of cultural heritage microbiology and mostly limited to the 16S rRNA gene, despite the fact that it is a multi-copy gene, what limits the microbial quantification by the genome size and type of the detected microorganisms (Masco, Vanhoutte, Temmerman, Swings, & Huys, 2007).

Mycosporine-like amino acids (MAAs) are a family of intracellular compounds biosynthesized by the shikimic acid pathway to synthesize aromatic amino acids (Wada, Sakamoto, & Matsugo, 2015). They have anampholyte nature and high denaturation temperature with water-soluble properties (Bozkurt & Kara, 2017) and are expressed under biotic and abiotic stresses,

e.g., solar radiation (Bhatia et al., 2011) and extreme conditions (Rosic & Dove, 2011). At times, MAAs can function as antioxidants and as osmolytes (Shu, Lee, & Jung, 2003). Because of their low molecular weight, MAAs such as mycosporine-glycine, shinorine and porphyra-334 can be synthesized quickly in response to light, nutrients, or temperature stress (Korbee, Figueroa, & Aguilera, 2006). High photosynthetically available radiation and UV exposure are the strongest inducing factors (Moisan, Goes, & Neale, 2009). Key genes related directly or indirectly to the MAAs synthase pathway would be promising single-copy molecular markers to detect SIAb from stone surfaces.

The qPCR technique's key to success is applying suitable molecular markers specific to a target group of organisms. The current study aimed to establish a time- and cost-effective technical approach to detect the potential biodeteriorant SIAb on cultural heritage stone structures and archeological sites, through: 1) development of SIAb specific molecular markers based on a comparative genomic approach conducted on two confirmed Geodermatophilaceae genomes, namely: *Blastococcus saxobsidens* Urzì et al. 2004 (Chouaia et al., 2012) and *Geodermatophilus obscurus* Luedemann 1968 (Ivanova et al., 2010); 2) validation of the proposed specific molecular markers on samples that were previously collected from two prehistoric Egyptian archeological sites (Djoser and Lahun pyramids).

3.2 Materials and Methods

3.2.1 Stone-inhabiting Actinobacteria specific marker identification

The MAAs synthesis pathway was the primary candidate to fulfill the study objective. Using STRING online tools (Szklarczyk et al., 2019) along with previous reports, a more comprehensive pathway was constructed using the two confirmed Geodermatophilaceae SIAb species in this database, namely: *B. saxobsidens* DD2 (BLASA) and *G. obscurus* DSM 43160 (Gobs). Candidate genes were extracted from the genomes using Geneious Prime (Kearse et al., 2012) and aligned with copies from Actinobacteria species for qPCR primer design.

The candidate genes Phospho-2-dehydro-3-deoxyheptonate aldolase synthase II (*aroF*) and Chorismate mutase (*cm2*) were extracted from *B.*

saxobsidens and blasted into the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All the qPCR primers were designed using Geneious primer, and calibrated using conventional PCR (cPCR) on five Actinobacteria species isolated from stone, three species belong to Geodermatophilaceae (*B. saxobsidens* DSM 44509, *G. carrarae* DSM 44511, and *Modestobacter* sp. DSM 44446) provided by Prof. Clara Urzì (Messina University, Italy), and two strains belong to Micrococcaceae *Kocuria sediminis* Bala et al. 2012 DPS08B (99.2% of BLAST similarity to GenBank: NR_118222) and *Micrococcus luteus* (Schroeter 1872) Cohn 1872 DPS12B (99.4% of BLAST similarity to GenBank: NR_075062) isolated from our previous work on Djoser pyramid area (see Chapter I). Detailed information on the primers can be found in Table 3.1. DNA extraction from the bacterial strains was performed using PureLink® Genomic DNA kit (Cat# K182001, Thermo Fisher Scientific, USA) according to the kit protocol. DNA quality was checked using 1% (w/v) agarose gel electrophoresis, visualized by pre-added RedSafe® dye under UV light, and quantified using Quantus™ Fluorometer (Promega, USA).

The cPCR reactions were performed using MyTaq™ Red Mix (Cat# BIO-25043, BioLine, UK) kit. In each 25 µl reaction tube included 5 pmol of each primer, and 40 ng on template DNA were added. The amplification was carried out using Techne™ 512 thermocycler (Techne, UK). PCR programs were adjusted according to the primer pair melting temperature (Tm) as follows: the first denaturation step at 95 °C for 3 min was followed by denaturation at 95 °C for 20 s, annealing was set according to each primer pair (Table 3.1), extension at 72 °C for 30 s. The last three steps were repeated 34 times, with a last extension 72 °C for 5 min. PCR products were tested using 1.5 % agarose gel electrophoresis, as previously described.

3.2.2 Stone-inhabiting Actinobacteria qPCR marker calibration and testing

Extracted environmental DNA (eDNA) from 12 archeological samples collected evenly from two pyramids (Djoser and Lahun pyramids) from a previous study (see Chapter I) were used for the newly developed qPCR markers validation. The universal primers for 16S rRNA (De Gregoris, Aldred, Clare, & Burgess, 2011) and the Actinobacteria specific 16S rRNA (Yang et al., 2015) along with

the newly designed primers were calibrated and tested using the following recipe and qPCR conditions: 5 pmol of each primer, 1x of SensiFAST™ SYBR® Lo-ROX Kit (Bioline, UK), 1 ul of eDNA and H_2O up to a total volume of 20 µl were prepared for each sample.

The qPCR was performed using Stratagene Mx3000P qPCR machine (Agilent Technologies, USA), and was set to the default of normal 3-steps standard thermal profile, while the annealing temperature was adjusted to 57 °C for *aro*F and *cm*2. The marker efficiency to amplify SIAb (M_{SIAb}) was evaluated for each sample using the following equation: M_{SIAb} = relative Actinobacteria CT value (CT16S/CTAct) / relative marker CT value (CT16S/CTmarker); where the CT (cycle threshold) values are for the 16S universally amplified bacteria (CT16S), 16S amplified Actinobacteria (CTAct), and amplified bacteria with a gene copy of the tested marker (CTmarker). Nomenclature of bacteria follows NCBI Taxonomy Browser (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Root).

Table 3.1 qPCR primers applied to detect SIAb in samples collected from archeological locations from Djoser and Lahun pyramids of Egypt based on *Blastococcus saxobsidens* DD2 genomic data (Actinobacteria, Geodermatophilaceae). For each primer, the following information is given sequence, melting temperature (Tm), expected length of the amplified product (bp), target gene, and reference if previously published.

Organism group	Primer name	5'-Seq-3'	Tm	bp	Gene	Reference
Universal	926F	AAA CTC AAA KGA ATT GAC GG	50	136	16Sr RNA	De Gregoris et al. (2011)
	1062R	CTC ACR RCA CGA GCT GAC				
Actinobacteria	Act664F	TGT AGC GGT GGA ATG CGC	60	277	16S rRNA	Yang et al. (2015)
	Act941R	AAT TAA GCC ACA TGC TCC GCT				
Actinobacteria/ proteobacteria	aroFqF	GCG CAC ATC GAC TTC ATC TC	57	283	*aro*F	Current study
	aroFqR	CGA AGT GCC GGG TCT TGT AG				
Actinobacteria	CmutqF	GTA ACA ACA GCA CTG GCA GC	57	237	*cm*2	Current study
	CmutqR	CGA TCT CGT CTA TCC GCT CC				

3.3 Results

3.3.1 Stone-inhabiting Actinobacteria specific marker identification

The pathway analysis showed that both the MAAs and shikimate pathways (i.e., biosynthesis of folates and aromatic amino acids) starts by stereospecific condensation of phosphoenolpyruvate (PEP) and D-erythrose-4-phosphate (E4P) to give rise to Phospho-2-dehydro-3-deoxyheptonate (DAHP) using Phospho-2-dehydro-3-deoxyheptonate aldolase (DAHP synthase), and then convert to 3-dehydroquinate (DHQ) by 3-dehydroquinate synthase (*aro*B) gene. Followed by either 3-dehydroquinate dehydratase (*aro*Q) to redirect the DHQ to 3-dehydroshikimate for shikimate pathway; or O-methyl transferase (OMT) to redirect the DHQ to deoxygadusol for the MAAs pathway (Fig. 3.1).

The *aro*B and *aro*Q genes were found not only in the two SIAb genomes but further in many other organisms (most of the recorded bacteria in STRING database, in addition to fungi and plants). But, based on this database, the OMT was not found in the two SIAb genomes, which disqualifies those genes as potential SIAb molecular markers. The DAHP synthase gene was found in duplicate as DAHP synthase I (*aro*G) and II (*aro*F), the latter of which was found only in few species belonging to Proteobacteria, Actinobacteria, Chlamydiae-Verrucomicrobia group, Fibrobacteres-Acidobacteria group, *Desulfurispirillum indicum* Rauschenbach et al. 2011, and some eukaryotic organisms. The Actinobacteria were the highest in terms of similarity and number of species when *aroF* from *B. saxobsidens* was used as an inquiry (Fig. 3.2).

Based on the *aroF* gene, an interactive gene network was inquired from STRING for the two SIAb genomes, where a similar pattern of genes was found. An additional candidate from the same pathway was detected for the two SIAb genomes, namely, Chorismate mutase, which was found in two homologs, homolog I (*cm*1) and II (*cm*2). Like *aro*F, the *cm*2 was found exclusively in Actinobacteria and few species from Proteobacteria (e.g., *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919; Fig. 3.2). Based on *E. coli* co-expression data from the same database, *aroF* was found to co-express with *cm2* (known as *phe*A in *E. coli*; r-value = 0.85).

3.3.2 Stone-inhabiting Actinobacteria qPCR marker calibration and testing

The qPCR primers were optimized for single band amplification using cPCR approach on the five bacterial species. For *aroF*, a single specific band was obtained for all species, the *Geodermatophilus carrarae* showed a weaker amplification what might be probably due to a variation in the primer target region; while *cm2* showed multiple bands for *Modestobacter* sp. and *Kocuria sediminis* that were decreased to one band after raising +2 °C Tm (Fig 3.3).

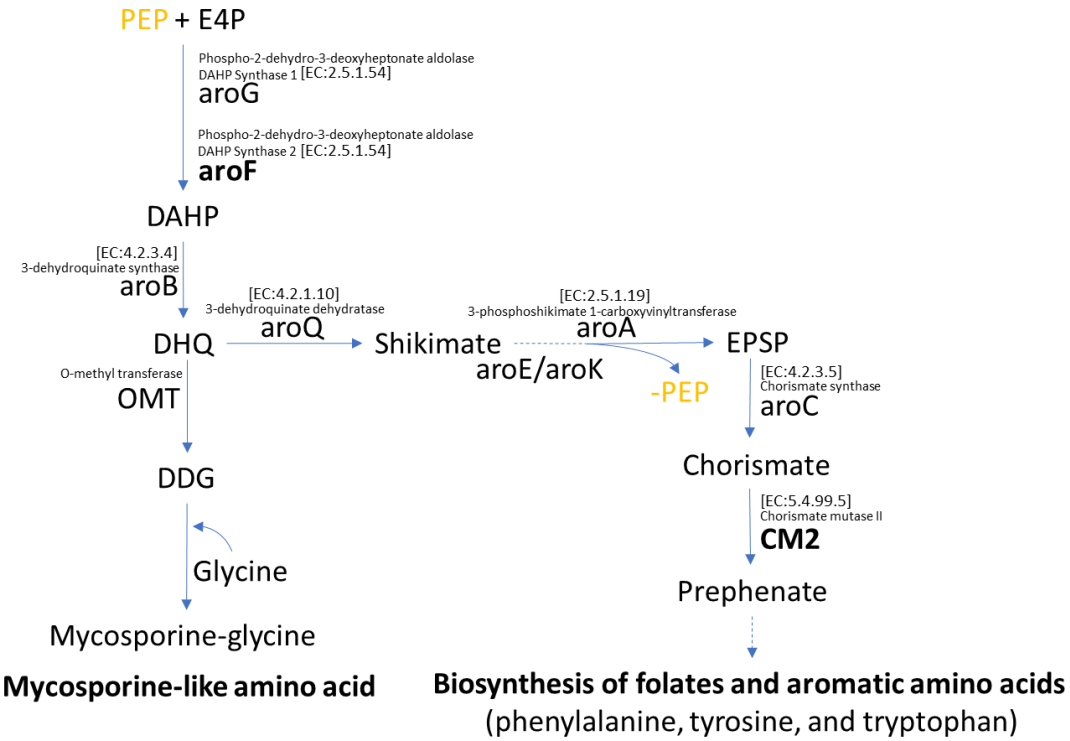

Fig. 3.1 Mycosporine-like amino acids and shikimate (biosynthesis of folate and aromatic amino acids) schematic pathway based on literature and STRING information. Potential candidate genes identified from one of the two SIAb, *Blastococcus saxobsidens,* are written in bold.

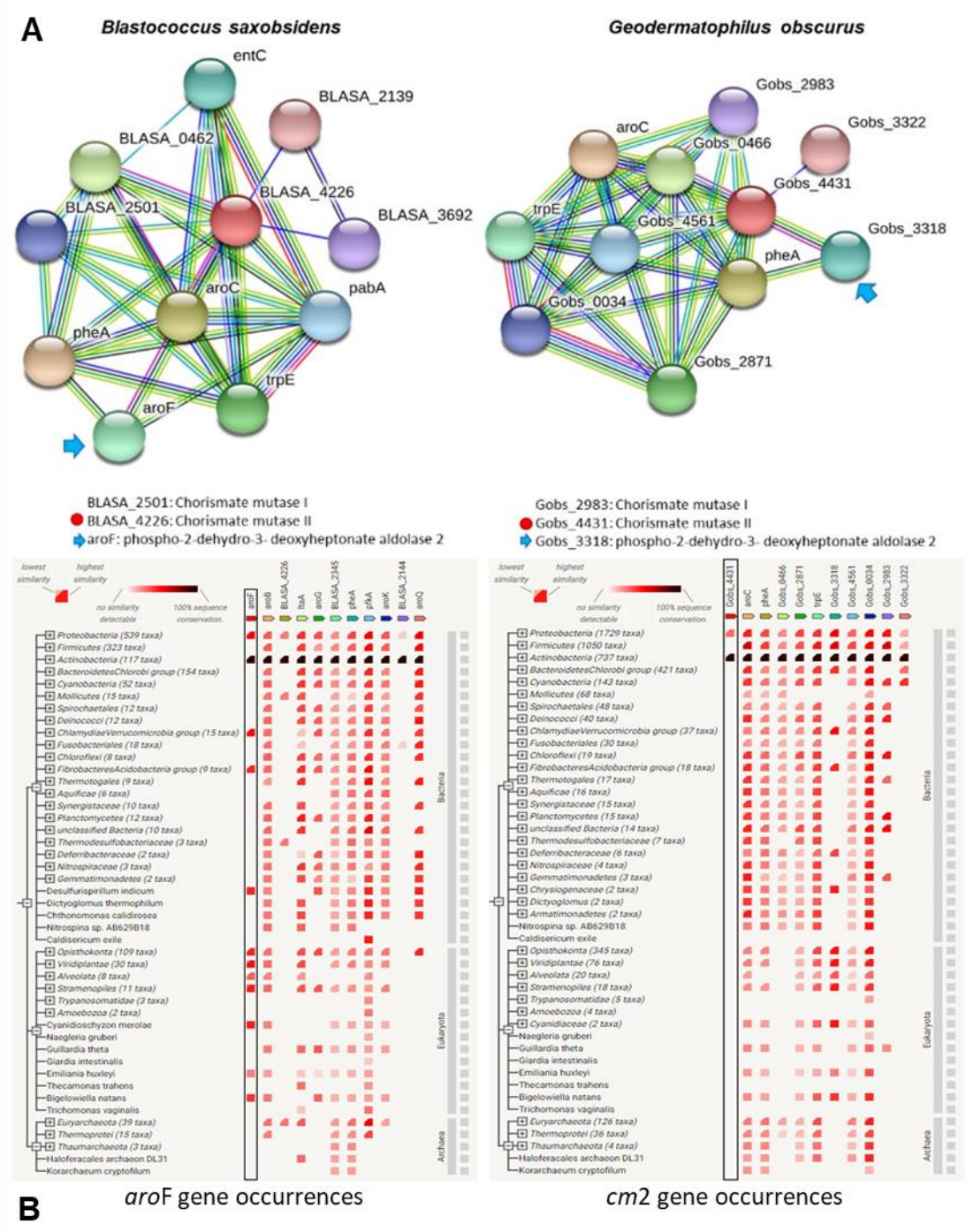

Fig. 3.2 Gene interaction and occurrences for *aro*F and *cm*2 genes in two SIAb species (*Blastococcus saxobsidens* and *Geodermatophilus obscurus*). (A) Gene interaction network visualized based on Chorismate mutase gene for the two stone-inhabiting bacteria. Blue arrow points to the *aro*F gene, while the red circle represents the *cm*2 gene. (B) The candidate genes occurrence graph along with neighbor genes and homologs from the same pathway among different organisms based on the STRING database. The search targeted the *B. saxobsidens* DD2 (BLASA) *aro*F gene (left) and the *G. obscurus* DSM 43160 (Gobs) *cm*2 gene coded as Gobs_4431 (right).

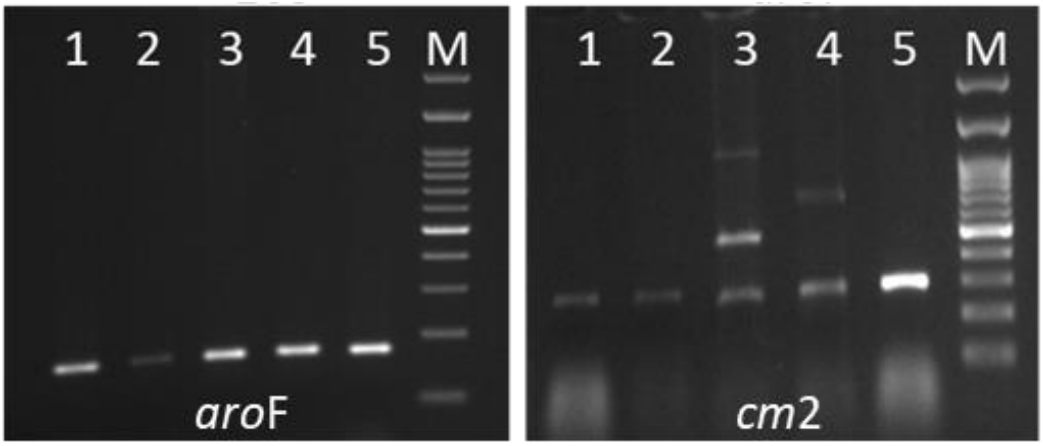

Fig. 3.3 Agarose gel electrophoresis shows the amplification of *aro*F and *cm*2 candidate genes based on the primer manufacturer melting temperature for: *Blastococcus saxobsidens* (1), *Geodermatophilus carrarae* (2), *Modestobacter* sp. (3), *Kocuria sediminis* (4), and *Micrococcus luteus* (5). The high-density band in the DNA marker ladder (M) = 500 bp.

The universal 16S rRNA qPCR primer was amplified successfully in all the tested samples, with CT values ranging from 21 to 24, with an average value of 22. The qPCR primers of both genes (*aro*F and *cm*2) were amplified correctly. The *aro*F recorded CT values ranged from 27 to 36, with an average value of 34, while the *cm2* recorded CT values ranged from 33 to 38, with an average value of 35. All regions showed a high specificity with a single peak by the melting curve test, and it was confirmed as a single band at the expected size by 1.5% agarose gel electrophoresis, as shown in Fig. 3.4, and generally varied among the collected samples. The estimated M_{SIAb} was 1.01±0.04 and 0.98±0.05 for *aro*F and *cm*2, respectively, reflecting an equal efficiency of the two potential markers as the Actinobacteria specific 16S rRNA primers to amplify the Actinobacteria successfully. The current results support the *aro*F and *cm*2 potentiality as molecular markers to detect SIAb specimens' presence in samples prior to proceeding with further analysis (e.g., metagenomics and metabarcoding analyses).

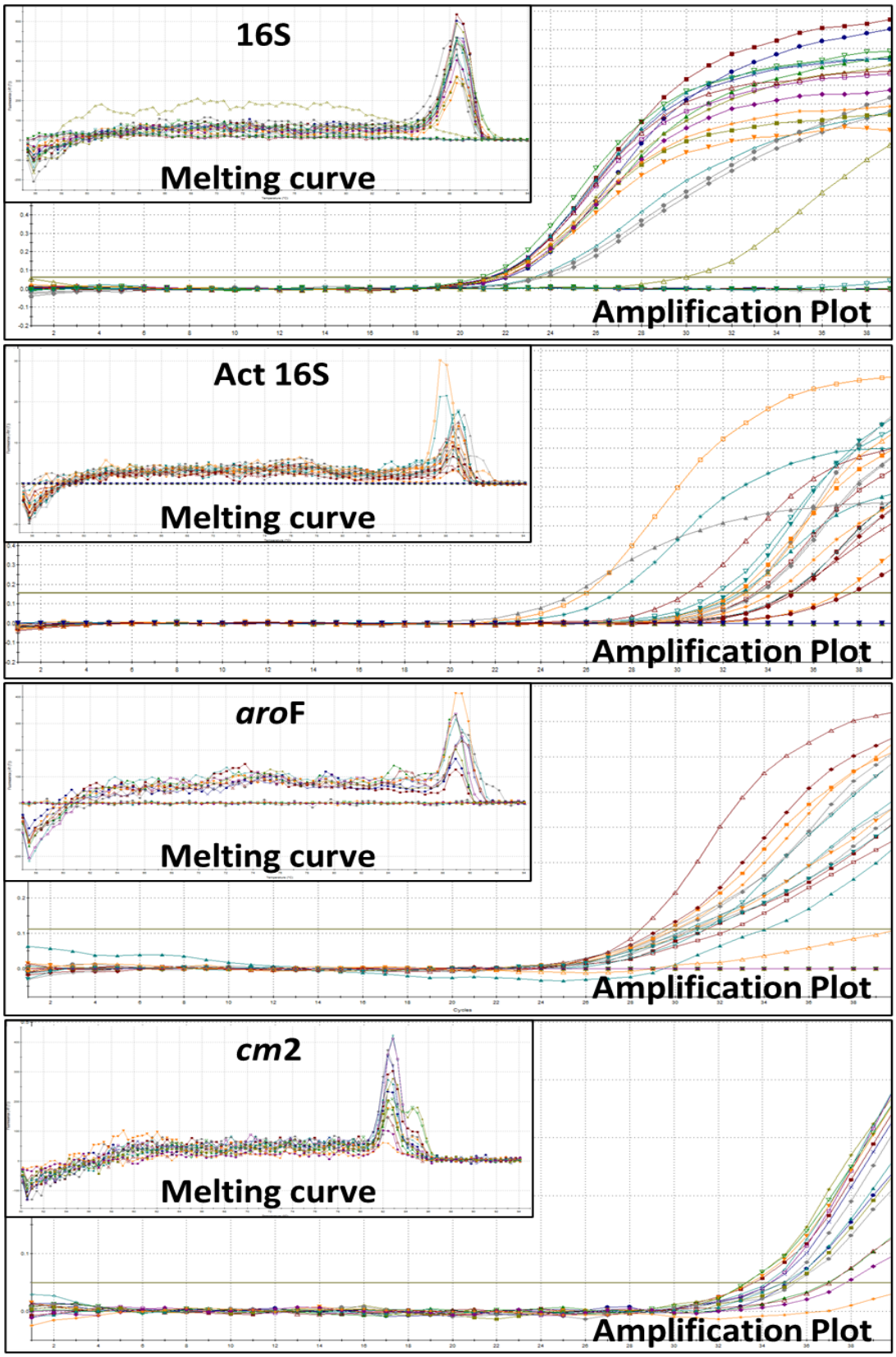

Fig. 3.4 qPCR amplification plots and melting curves for 16S rRNA universal qPCR primer (16S), Actinobacteria specific 16S rRNA primer (Act 16S), *aro*F and *cm*2 using the eDNA extracted from cultural heritage material.

3.4 Discussion

DNA and RNA-based methods in biodeterioration of cultural heritage have gained much attraction over the past decade (Sterflinger, 2010; Otlewska, Adamiak, & Gutarowska, 2014; Piñar, Poyntner, Lopandic, Tafer, & Sterflinger, 2020). The primary use of these methods was detecting and characterizing microorganisms in environments that are notoriously hard to culture and where the inhabited microorganisms are often underestimated (Scheerer et al., 2009). The best methodological approach to identify and study a complex microbial community is through the combining of microscopy and molecular identification for culture-dependent microbes while using metagenomic approaches for culture-independent ones by the extraction of DNA and/or RNA directly from the substratum and/or biomass (De Leo, Iero, Zammit, & Urzì, 2012). Besides the difficulties of sample collection in a non-destructive method from culturally important sites (Urzì & De Leo, 2001) and the eDNA extraction from samples (Schneegurt, Dore, & Kulpa, 2003), the identification techniques can be expensive and time-consuming. For example, many researchers use Epifluorescence microscopy to detect the microbial community's viability in samples before proceeding with further analysis (e.g., Urzì & De Leo, 2001; Ricca et al., 2020). Therefore, to have a fast and reliable molecular method for surveying cultural heritage material for possible biodeterioration agents is required.

Actinobacteria demonstrated a great taxonomic diversity on stone surfaces and, despite the predominance of isolates of the genus *Streptomyces* Waksman & Henrici 1943, members of the genera *Nocardia* Trevisan 1889, *Rhodococcus* Zopf 1891, and *Geodermatophilus* have also been described (Groth, Vettermann, Schuetze, Schumann, & Sáiz-Jiménez, 1999). In addition to *Streptomyces* and *Nocardia*, *Micromonospora* Orskov 1923 species were also isolated from ancient stone from a tomb site in Tell Basta (Zagazig, Egypt; Abdulla, May, Bahgat, & Deweda, 2008). The Geodermatophilaceae SIAb species can withstand the harsh conditions of the outdoor stone surfaces (Gtari et al., 2012). As MAAs are compounds that can be quickly synthesized in response to abiotic stress, as well as light, nutrients, and temperature stresses (e.g., Bhatia et al., 2011; Rosic & Dove, 2011), exploring key genes related

directly or indirectly to the MAAs synthase pathway would be promising molecular markers to detect the adapted SIAb to the stone surfaces' harsh conditions.

Urzì et al. (2004) developed specific probes for the detection of *Geodermatophilus* and *Modestobacter* species colonizing rock surfaces by using fluorescence in situ hybridization technique. To the best of our knowledge, most of the qPCR protocols to detect and/or quantify bacterial communities in cultural heritage material were designed based on the 16S rRNA gene. For example, del Mondo et al. (2019) used the 16S rRNA to detect the whole bacterial content for subaerial biofilms on stone monuments. While previously, Salazar et al. (2006) developed and reported the detection and quantification of Geodermatophilaceae species from stone samples using family-specific qPCR primers to detect its presence on stone surface from Spain; this approach was developed mainly on the quantification of those species using the 16S rRNA gene only. The main issue with the 16S rRNA based quantification is its multi-copy nature in many bacteria. Even though this does not affect the microbial species identification (Ibal, Pham, Park, & Shin, 2019), it still requires prior knowledge of the bacterial content to correct the copy number per bacterial genome (Masco et al., 2007). Moreover, very limited resources of confirmed SIAb genomes are available according to prokaryotic genome databases (e.g., STRING). Therefore, 16S rRNA may not provide the best tool for quantifying bulk eDNA microbial groups such as the SIAb.

In the current study, two specific genes (*aro*F and *cm*2) related to the production of MAAs were examined as potential molecular markers to detect the SIAb. Comparing both markers to the Actinobacteria specific primers proved the two markers' efficiency to amplify Actinobacteria detected in the eDNA samples. The developed approach was designed to rapidly detect the presence of SIAb but not for their absolute quantification. The use of the qPCR is for its sensitivity to detect very low concentrations, as qPCR achieves low limits of detection (1 x 10-7 ng/µl) and higher detection rate employed in eDNA for laboratory and field samples than cPCR (Xia et al., 2018). The protocol is developed as a preliminary test for archeological stone samples and cultural heritage material prior to metagenomic and/or culture-dependent analyses to reduce cost and time, respectively.

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General Discussion

1. Hyper-arid related microbial diversity

Besides physical and chemical erosion to stone surfaces, microorganisms plays a key role in the stone deterioration and widely known as biodeterioration (Warscheid & Braams, 2000; Liu, Koestler, Warscheid, Katayama, & Gu, 2020). Both erosion and biodeterioration influence irreversible stone transformation and decay causing significant damage to monuments of cultural value and historical significance (e.g., Urzì, De Leo, de Hoog, & Sterflinger, 2000).

The step-pyramid (Djoser) and the mud-pyramid (Lahun), each is a prehistoric archaeological remain forming part of a bigger mortuary complex surrounded by decoration and ceremonial structures. Both are the largest and oldest pyramids of the ancient Egyptian civilization located in the Memphis necropolis, on the west side of the Nile valley (southwest the modern Cairo, capital of modern Egypt). Outside the Nile valley, the geological aspect changes dramatically from clay soil on the banks of the Nile river to sandy soil, which is light, warm, dry and tend to be acidic with low nutrients availability (Mahmoud, Alazba, Adamowski, & El-Gindy, 2015). Hyper-arid climate, high exposure to sun light (UV), and high fluctuation of temperature between day and night are the prominent macroenvironmental features characterizing the surveyed pyramidal complexes. The surveyed communities of both bacteria and fungi showed variable number of phyla that coexist in the sampling area, such variation reflect the heterogenic nature of the studied microenvironment.

At bacterial level, Actinobacteria (families Geodermatophilaceae and Micrococcaceae) and Bacilli (families Bacillaceae and Planococcaceae), were the most abundant classes for both pyramids, followed by a significantly smaller percentage of Bacteroidia, Chloroflexia, and Gammaproteobacteria. In a similar environment, metagenomics among desert varnish (i.e., dark rock coatings) samples from Anza Borrego, Mojave deserts and Johnson Canyon (California, U.S.A.), Jabal Yatib (Saudi Arabia), Negev desert (Israel) was applied. The most abundant shared OTUs among all sites were Actinobacteria (families Geodermatophilaceae and Rubrobacteraceae) and Alphaproteobacteria (families Acetobacteraceae and Sphingomonadaceae), with smaller contributions of Cyanophyceae (families Phormidiaceae and Xenococcaceae; Lang-Yona et al., 2018). Similarly, from Namib desert (South Africa), the hyper

arid desert soil niche community was investigated using metagenomics. Classes Actinobacteria (orders Actinomycetales and Rubrobacteriales), Alphaproteobacteria (orders Caulobacterales, Rhizobiales and Sphingomonadales), and unassigned classes to Cyanobacteria, were the most abundant classes and orders among the sampled locations (Vikram et al., 2016). Compared to our results, besides the Actinobacteria, Bacteroidetes and Proteobacteria that are known as typical hot desert phyla across the world (Makhalanyane et al., 2015), Cyanobacteria (class Cyanophyceae) were not found dominating the sampled pyramidal sites, in contrast to the Firmicutes (class Bacilli). Thus, the abundant presence of the Bacilli class on both pyramids, should not be attributed to the surrounding environmental factors but maybe to the nature of the stone material of the pyramids. Both pyramids are formed from different materials, one was built from limestone (Djoser) and the other from mud (Lahun). This would explain the reversed percentage values of Actinobacteria and Bacilli classes between both pyramids. Species belonging to Bacilli class are known of breaking down poorly degradable compounds (e.g., drill mud; Struchtemeyer, Davis, & Elshahed, 2011) and have been extensively identified on stone buildings (e.g., Blazquez, Lorenzo, Flores, & Gómez-Alarcón, 2000; Heyrman & Swings, 2001; Laiz, Pinar, Lubitz, & Saiz-Jimenez, 2003; Kiel & Gaylarde, 2006).

At fungal level, the class Dothideomycetes (family Pleosporaceae) was the most abundant for both pyramids, followed by Eurotiomycetes (family Trichomeriaceae). In Lang-Yona et al. (2018) study, the desert varnish microbiome mainly comprises bacteria, with minor contributions of archaea and fungi, however, the dominant fungal class was Dothideomycetes. Similar discoveries were also found by Kuhlman, Venkat, La Duc, Kuhlman, & McKay (2008) from rock varnish microbiome from Atacama Desert (Chile). In Saudi Arabia, a country with similar climate to the pyramid's location, soil samples from different locations were studies using ITS2 metabarcoding technique, the dominant fungal classes were Eurotiomycetes, Pezizomycetes and Sordariomycetes, across all samples (Moussa, Al-Zahrani, Almaghrabi, Abdelmoneim, & Fuller, 2017).

2. Microorganism inhabiting Djoser and Lahun pyramids

Stone-inhabiting microorganisms (e.g., bacteria, fungi, algae, lichens, and protozoa; Urzì et al., 2000; Scheerer, Ortega-Morales, & Gaylarde, 2009; Sanz et al., 2017; Pena-Poza et al., 2018) may be epilithic, chalcolithic, or endolithic and are mostly extremotolerant that are barely surviving and rarely reproducing (Rampelotto, 2013). Due to the species richness and dominancy varied between the two surveyed pyramids, the commonly recorded and highly represented species were the key features to defining the relevant stone-inhabiting bacteria (SIB) and rock-inhabiting fungi (RIF). However, the key was not applied with a confirmed and/or previously reported species and/or genera.

2.1 Stone-inhabiting bacteria

Actinobacteria is considered the dominant bacteria in subterranean habitats, such as caves and tombs (Cuezva et al., 2012). Actinobacteria demonstrated a great taxonomic diversity on stone surfaces and, despite the predominance of isolates of the genus *Streptomyces* Waksman & Henrici 1943, members of the genera *Nocardia* Trevisan 1889, *Rhodococcus* Zopf 1891, and *Geodermatophilus* Luedemann 1968 were also described (Groth, Vettermann, Schuetze, Schumann, & Saiz-Jimenez, 1999).

In our study, based on metabarcoding, the most abundant species belonged to the family Geodermatophilaceae, which was described as one of the most important stone-inhabiting Actinobacteria (Urzì et al., 2001; Sghaier et al., 2016). Two known species of the family Geodermatophilaceae, *Blastococcus aggregatus* Ahrens & Moll 1970, and *B. saxobsidens* Urzi et al. 2004 that cause an orange coloration, were identified from both pyramids. The latter was found to resist harsh environmental conditions (e.g., UV light, ionizing radiation, desiccation, and heavy metals; Gtari et al., 2012; Montero-Calasanz et al., 2015).

The species representing the family Micrococcaceae (class Actinobacteria) was *Kocuria rosea* (Flugge 1886) Stackebrandt et al. 1995 known as a common soil and water species, which was found previously in extreme environments such as heavily polluted waters, deep-sea sediments, and spacecraft surfaces (Coil et al., 2016).

Exclusive to Lahun pyramid, it was found a species belonging to the genus *Micromonospora* (family Micromonosporaceae, class Actinobacteria) which was previously reported from decayed stone (Ciferri, Tiano, & Mastromei, 2000). Moreover, species from the same genus were isolated from a tomb in Tella Baste (Zagazig city, Egypt) along with isolates from *Micropolyspora* Lechevalier et al. 1961 (synonym of *Nocardia* Trevisan 1889) and *Streptomyces* (Abdulla, May, Bahgat, & Deweda, 2008). Other isolates were best known as common environmental species of natural presence on soil (e.g., *Arthrobacter* Conn and Dimmick 1947; Eschbach, Möbitz, Rompf, & Jahn, 2003).

Concerning the highly represented families in both pyramids, the Planococcaceae, gram-positive bacteria with no known characteristics exclusive to all members of the family, dominate. However, the known isolated species was *Planococcus salinarum* Yoon et al. 2010, which was previously isolated from a marine solar saltern, and can grow *in vitro* at up to 13% w/v NaCl (Yoon, Kang, Lee, Oh, & Oh, 2010).

Confirmed SIB belong to the family Geodermatophilaceae can withstand the harsh conditions of the outdoor stone surfaces (Gtari et al., 2012). Based on metabarcoding, the most surveyed functional bacterial gene group from both pyramids was the amino acid transport and metabolism, which in bacteria was found associated with abiotic stress tolerance mechanisms (Batista-Silva et al., 2019).

One group is the mycosporine-like amino acids (MAAs), a family of intracellular compounds biosynthesized by the shikimic acid pathway to synthesize aromatic amino acids are expressed under abiotic stress, as well as light, nutrients, and temperature stresses (e.g., high UV exposure; Bhatia et al., 2011). Such pathways are potential candidates to understand the stone-inhabiting microorganisms and would be promising molecular markers to detect the adapted Actinobacteria to the stone surfaces' harsh conditions.

2.2 Rock-inhabiting fungi

By comparing our results with similar studies, several fungal genera detected in the Djoser and Lahun pyramids were previously reported from cultural heritage material. For example, *Alternaria* Nees, *Aspergillus* P. Micheli, *Caldosporium* Link*, Epicoccum* Link*, Fusarium* Link, *Mucor* Fresen, *Penicillium* Link and *Trichoderma* Pers. were reported among others from storeroom objects in the Tianjin Museum, China (Liu et al., 2018), Etruscan tombs in Italy and ancient tombs of the Baekje Dynasty in Republic of Korea (Caneva, Isola, Lee, & Chung, 2020). However, based on metabarcoding functional analysis, most of the detected fungi are naturally present in the soil, while some are mold, plant-pathogen species, and/or wood-inhabiting fungi (e.g., *Chaetomium globosum* Kunze).

Based on metabarcoding data, the family Pleosporaceae (class Dothideomycetes) was one of the most abundant families shared between both pyramids represented by phylogenetically proved black meristematic fungi or microcolonial fungi, also referred as RIFs, *Alternaria chlamydospora* Mouch. and *A. oudemansii* (E.G. Simmons) Woudenb. & Crous (Ruibal et al., 2009; Piñar, Poyntner, Tafer, & Sterflinger, 2019). Followed by the family Trichomerieace (class Eurotiomycetes) represented by an extremotolerant RIF known as *Knufia karalitana* Isola & Onofri (Isola et al., 2016). The most remarkable isolated fungal species was a black meristematic fungus, *Pseudotaeniolina globosa* De Leo, Urzì & De Hoog that was previously described as RIF (De Leo, Urzì, & de Hoog, 2003).

Pseudotaeniolina globosa is one of the uncommonly recorded RIF and was found exclusively on Djoser pyramid; only few accessions were reported previously from water-limited ecological. The Egyptian isolate was slimy and formed yeast-like colonies after 4-5 days of growth, which differentiate it from a micro-morphologically similar species known as *Sarcinomyces petricola* Wollenzien and de Hoog (Wollenzien, de Hoog, Krumbein, & Uijthof, 1997; De Leo et al., 2003). According to the NCBI taxonomy database, the genus *Pseudotaeniolina* is uncertainly positioned within the order Capnodiales (*incertae sedis*) with no family assigned. Black meristematic fungi or microcolonial fungi, also referred as RIF possess convergent morphological

and physiological characteristics; however, many subgroups of RIF are phylogenetically uncertain within the class Dothideomycetes (Ruibal et al., 2009). In comparison to the first known isolate of *P. globosa* from Italy (De Leo et al., 2003), the morphological measurements of the hyphal length and width on MEA media of the Egyptian isolate were smaller than the Italian, however, morphologically, the Egyptian produced single cells or asymmetrically septate cells after conidiogenesis which is a unique character to *P. globosa* species (De Leo et al., 2003). In the current study, based on five molecular markers (ITS, SSU, LSU, BT2, and RPB2), the Egyptian isolate was proved to be *P. globosa* and was clustered along with the Italian isolate within the Teratosphaeriaceae family, order Capnodiales with bootstrap value > 0.7 – 0.99.

Regardless of the morphological variation between both the Egyptian and Italian isolates, under abiotic stress conditions *in vitro* (i.e., pH, salinity, and temperature) the Egyptian showed tolerance to extreme conditions. Compared to all Ascomycota, Capnodiales, Dothideales, and Eurotiales (class Dothideomycetes) are the main halophilic and halotolerant orders (Gunde-Cimerman et al., 2009) and contain many extremotolerant species that can grow as epilithic or cryptoendolithic at high or low temperatures (Selbmann, de Hoog, Mazzaglia, Friedmann, & Onofri, 2005).

RIFs are melanized microorganisms with observed UV tolerance as they are often associated with exposed natural rocky substrates (Sterflinger, 2000; Gorbushina, 2007). Both isolates were melanized, their ability to with stand UV treatment was measured and recorded almost similar regrowth rates after exposure to UV, which was not surprising; melanin not only serves as an extracellular electron-dense granular layer or UV protector for black fungi and yeasts but also form an intracolonial matrix structure (Gorbushina, 2003).

3. Methodological achievements

3.1 Metabarcoding *versus* traditional isolation

Metabarcoding may face a serious challenge in the extracted DNA from arid and hyper-arid soil samples or cultural heritage material (Schneegurt, Dore, & Kulpa, 2003), in our case, bulking strategy was followed to increase the eDNA concentration and improve the metabarcoding library and sequencing. In the current study, microbial diversity was higher than expected compared to similar studies (e.g., Moussa et al., 2017; Lang-Yona et al., 2018).

Compared with the isolated culture-dependent bacterial species, the Geodermatophilaceae and Planococcaceae species were never cultured by the currently used media. Similarly, *Knufia karalitana* was never cultured in contrast to *Pseudotaeniolina globosa* that was only cultivated but not detected by the metabarcoding technique. Additionally, *Bacillus alkalitelluris* Lee et al. 2008, *B. persicus* Didari et al. 2013*, Planococcus salinarum*, and *Planococcus* sp. were detected only by metabarcoding which will need to be isolated for further investigation to examine their biodeterioration effect. Also a fungal species belonging to the family Sporomiaceae will need further isolation and identification. Therefore, different media compositions and protocols will be required, especially for the relevant and those unknown species that were found highly represented in both pyramids.

Even though the metabarcoding identification method cannot provide a biological isolate of the detected microbial species, but it is still more advantageous, when it comes to understand the behavior of an entire microbial community (Li, Zhang, He, & Yang, 2016); besides the traditional methods enable only the detection of ≤ 5% of the total microbial community (Dakal & Arora, 2012).

Thus, the best methodological approach to identify and study a complex microbial community in certain substratum and/or biomass should follow a two-approach protocol, one is through microscopy and molecular identification for culture-dependent microbes and the other through metabarcoding for culture-independent microbes.

3.2 Stone-inhabiting Actinobacteria qPCR markers

DNA- and RNA-based methods in biodeterioration of cultural heritage were developed and applied in many previous reports (e.g., Sterflinger, 2010; Otlewska, Adamiak, & Gutarowska, 2014; Piñar, Poyntner, Lopandic, Tafer, & Sterflinger, 2020). The difficulties of sample collection in a non-destructive method (Urzì & De Leo, 2001) and challenges in eDNA extraction from such small amounts (Schneegurt et al., 2003), requires a fast and reliable low-cost molecular method for surveying cultural heritage material for possible biodeterioration agents prior to any sophisticated analysis. Two single-copied specific genes (*aro*F and *cm*2) related to the production of MAAs were examined as potential molecular markers to detect the stone-inhabiting Actinobacteria (SIAb). The newly developed markers were compared to the 16S Actinobacteria specific primer and proved the efficiency to amplify Actinobacteria detected in the eDNA samples. The protocol was developed as a preliminary test for archeological stone samples and cultural heritage material prior to metagenomic and/or culture-dependent analyses to reduce cost and time, respectively.

The developed approach was designed to rapidly detect the presence of SIAb from small sampling amount at very low DNA concentrations use of the qPCR, but not for their absolute quantification. The qPCR technique achieves low limits of detection (1 x 10-7 ng/µl) and higher detection rate employed in eDNA for laboratory and field samples than conventional PCR (Xia et al., 2018).

To the best of our knowledge, most of the qPCR protocols to detect and/or quantify bacterial communities in cultural heritage material and rock-surfaces were designed based on the 16S rRNA gene. For example, specific probes were designed for *Geodermatophilus* and *Modestobacter* Mevs et al. 2000 emend. Qin et al. 2013 species (Urzì, La Cono, & Stackebrandt, 2004), the quantification of Geodermatophilaceae species from stone samples (Salazar, Valverde, & Genilloud., 2006), and determine the bacterial content for subaerial biofilms on stone monuments (del Mondo, de Natale, Pinto, & Pollio, 2019). The 16S rRNA based identification is not affected by its multi-copy nature (Ibal, Pham, Park, & Shin, 2019); however, the quantification requires prior knowledge of the bacterial content to correct the copy number per bacterial

genome (Masco, Vanhoutte, Temmerman, Swings, & Huys, 2007). However, fully characterized SIAb genomes are still not available (e.g., STRING database). Thus, a multicopy 16S rRNA gene may not provide the best tool for quantifying bulk eDNA microbial group, but rather a single copy one.

Our prospective analysis will target the genomic signatures of the extremotolerance characters of the DPS10, to accurately detect the transcriptional responses and metabolic suspension and reactivation under the fluctuation of water availability and its extreme ability to maintain viability in a high salinity medium. Which will be achievable with more detailed comparison at genomic, transcriptomic, and metabolic levels using NGS-based approaches.

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CONCLUSIONS

The main conclusions obtained of this thesis can be summarized as follows:

1. The present study sheds light on the knowledge of the microorganism communities that inhabit and can cause serious damage to outdoor stone monuments specially pyramids as it is a new study of their kind in Egypt on prehistoric culture heritage objects especially from the pharaonic era.
2. Combining the metabarcoding approach with the traditional isolation method for culture-independent and culture-independent microbes, respectively, helped to effectively develop a multi-step strategy for surveying and identifying the diversity of the microbial communities present on Djoser and Lahun pyramids as outdoor monuments.
3. The microbial diversity observed in both pyramids is higher than expected, considering the harsh conditions of the sampling sites.
4. The detected black meristematic fungi or RIF highlight the role of the genera *Alternaria*, *Knufia* and *Pseudotaeniolina*, in addition to the SIB of the family Geodermatophilaceae as potential bio-deteriorating agents of the two pyramids studied.
5. Additional stone-inhabiting microorganisms that dwell on the surface of stone monuments were detected and require attention as more investigation to assess the contribution of those microorganisms to the deterioration of cultural heritage objects is needed, as well as the evaluation of possibilities for their control.
6. The metabarcoding expands the knowledge of new types of microbial metabolism occurring in these habitats, what helps to develop molecular markers to detect SIAb on cultural heritage material.
7. The development of new identification methods provides a broader understanding of the diversity of organisms present on outdoor monuments and may expand the knowledge of new species or biovariants.

8. The harsh local climatic conditions of the surveyed monuments forces selection of stone-inhabiting microorganisms favoring extremotolerant biovariants and genotypes.
9. The surveyed alterations on the two studied pyramids allowed the demonstration of specific microorganisms, however, knowledge of the precise mechanisms of inhabiting the monuments and its role in biodeterioration is poorly studied, which demands more attention and additional investigation (e.g., the application of transcriptomics and genome-wide association studies).